

Two cheers for the multilateral malaria initiative

A major health crisis is being belatedly addressed by those who can make a difference. Real progress can be anticipated — provided participants act with regard for each others' constraints and experience.

Championing the fight against a single disease when there are so many other deserving causes is an uncomfortable affair. But there is a growing international consensus, shared by this journal, that malaria deserves special consideration. And what is good for malaria is also likely to be good for tropical disease research in general, and for support for research and health in developing countries.

Malaria kills more than one million people every year, some 300 million people have the disease, and one-third of all humanity lives in zones where they risk catching it. At the same time, resistance of the parasite to existing drugs is burgeoning, while the pharmaceutical industry has virtually abandoned research and development of new drugs and vaccines. Funding of the research that could bring new tools is shamefully low — less than \$100 million a year — in particular when compared to funding for other less significant diseases such as asthma.

In anybody's reasonable terms, this combination of calamities amounts to a 'crisis'. Credit must therefore go to the world's major research donor and to development agencies, as well as industry, for recognizing, albeit belatedly, this challenge and deciding that action is urgently needed. Over the past six months, they have together created a forum, the Multilateral Initiative in Malaria (MIM), of considerable weight. While sorting out their priorities, members of the MIM are encouraging industrialists to reverse their withdrawal from the field.

The prominent role of the United States, and Harold Varmus, the director of the National Institutes of Health (NIH) in particular, has been a key factor in the success of the movement. But the United States needs to be careful that it does not derail progress by isolating itself from its international partners. At a meeting of the MIM held last week in The Hague (see page 219) the United States upset several of its partners by springing on them radical proposals under which, for example, the various agencies that fund research would put money into a common pot, and create a joint structure to peer-review and choose

research projects for financing.

Varmus is right to demand greater imagination from funding agencies. But the United States is a relatively new player in African research, and US funding for malaria has declined over the past decade. This makes it all the more necessary for the United States to address legitimate objections to new mechanisms in full consultation with its partners. It should also be seen to appreciate the constraints under which many agencies work. Only in that way can progress be made.

It would be a shame if the United States were to reduce in any way its interest in MIM as a result of impatience to bring about change in mechanisms for administering collaborative research. Moreover, such narrow issues of research management are ultimately secondary to the bigger question of how to attract substantial new funding for malaria research. There is little incentive to discuss new funding mechanisms for research projects when there are no extra dollars on the table.

That this basic fact now seems to have been taken on board by MIM is heartening news. MIM must now identify priorities and strategies and also the ways in which these can dovetail with control programmes and capacity building in Africa. Malaria researchers themselves have a responsibility here also. In the past, the field has too often been characterized by infighting among scientists, and exaggerated promises as to the possibilities and time-scale of progress — the World Health Organisation has been particularly guilty of the latter. Politicians are reluctant to fund areas where the messages are unclear.

MIM's urgent task is to harness a diverse and powerful talent to develop realistic strategies that will convince politicians and funding agencies that extra investment in malaria research is a plausible means of alleviating the plight of the millions afflicted by the disease. MIM has taken upon itself to be the global body representing malaria research. It must now knuckle down to the hard work that will be needed if it is to fulfil this responsibility and make a serious impact on malaria. □

Martian life to be avoided

Sending people to Mars is a bad idea.

Any day now it's going to happen: some politician will stand up and exhort the United States, single-handedly or otherwise, to put human life on Mars. Forces within the National Aeronautics and Space Administration (NASA) want it. Some of the public would be enthralled, not least at the sheer risk. Engineers would relish the challenge. Industry would love the money. But given that such expenditure would be misdirected, one can only hope that, this time round, the proponent will not be as influential as a US president.

Some will say that only with direct human involvement in exploration will political support for serious planetary exploration be forthcoming. That reflects a poor view of the politicians' understanding of the public, let alone their sense of responsibility with taxpayers' money — excellent science on Mars is eminently do-able without humans, and cheaply, as Pathfinder is demonstrating. The public's enthusiasm

for manned exploration in space is fickle and the inspiration shallow — witness the legacy of the Apollo programme, if only there was one.

Strikingly, the events of recent days show that the public's enthusiasm for unmanned exploration can be significant. More to the point, they also show the extent and great potential of developments in robotics and remote measurement, and what can be achieved with engineering subtlety. That subtlety — as in the multiple gravitational slingshots of past planetary missions — is where NASA has undoubted strengths. Others too have provided examples to be followed, such as the returns of samples that took place during the unmanned Soviet Luna missions in the 1970s. That tradition of engineering ingenuity in the pursuit of science, rather than astronauts delivered to a lethal and barren landscape at huge cost, provides the appropriate inspiration to be followed for the foreseeable future of planetary surveys. □



Asian economies lead increase in carbon dioxide emissions

[LONDON] Global carbon dioxide emissions from the burning of fossil fuels continued to increase throughout 1996, according to statistics compiled by the London-based World Energy Council (WEC). The highest percentage rises came from the Asia-Pacific region, including India, China and the newly industrializing 'tiger' economies, as well as the Middle East.

The statistics will inevitably strengthen calls from the United States for the industrializing countries to be included in a proposed legally binding treaty to reduce carbon dioxide emissions. The treaty, due to be negotiated in Kyoto, Japan, in December, is aimed at reducing the impact of man-made global warming.

Carbon dioxide emissions in North America increased by 3 per cent last year to 1.76 billion tonnes of carbon (the increase from 1994 to 1995 was less than 1 per cent). The rising emissions are likely to strengthen pressure on the United States to sign up to clear reduction targets.

But emissions also increased in the European Union (EU), which for the first time since 1992 failed to maintain carbon dioxide emissions below 1990 levels. This will strengthen American scepticism of an EU pledge to reduce emissions by 15 per cent by 2010.

At the end of 1996, says the report, global emissions of carbon dioxide stood at 6.5 billion tonnes — 6.4 per cent higher than in 1990 and 2.7 per cent higher than in 1995.

The WEC, whose members represent national energy industries, compiled its report from data provided by governments and energy companies on emissions from three fossil fuel sources: coal (2.4 billion tonnes of carbon), oil (2.8 billion tonnes), and natural gas (1.3 billion tonnes).

The Asia-Pacific region — excluding Japan, Australia and New Zealand — registered a 37 per cent increase in emissions from 1990. They now stand at just over 1.5 billion tonnes a year, up 5.5 per cent from 1995.

In sharp contrast, emissions from eastern Europe continued to decline, although the 2.5 per cent drop from 1995 to 1996 was much smaller than in previous years. Emissions in some countries, notably the Czech Republic, Poland, Hungary and Romania, have begun to rise again.

After three consecutive years of falling emissions up to 1994, EU countries recorded their second successive increase — the first time the 1990 threshold had been crossed. At the end of 1996, the EU's 15 member countries together were emitting 957 million tonnes of carbon a year, 0.8 per

	'90	'95	'96	'90-96 % change	'95-96 % change
U.S.	1618	1706	1759	+8.7	+3.1
Asia-Pacific	1126	1465	1546	+37	+5.5
France	112.6	109.4	111.0	-1.6	+1.5
Germany	281.9	254.7	260.1	-7.8	+2.1
Italy	120.9	121.8	121.9	+0.8	+0.1
U.K.	170.0	163.5	168.3	-1.0	+2.9
EU Total	949	936	957	+0.8	+2.3

Figures in millions of tonnes of carbon

cent higher than in 1990.

Most of the additional emissions came from Germany, the United Kingdom and Denmark, widely considered to be three of Europe's most environmentally conscious countries. Michael Jefferson, the WEC's deputy secretary general, believes that, for Germany and the United Kingdom at least, the 'honeymoon' caused by the shutdown of polluting industries now seems to be over.

According to Jefferson, the statistics suggest that emissions may be on the rise. But British government officials disagree, saying the increase is within predicted forecasts and of no particular significance.

There is, however, little doubt that the statistics will be a setback to Europe's efforts to seize the moral high ground in the race to slow down man-made global warming. They may also fuel doubts about the United Kingdom's ability to meet its even more ambitious promise of a 20 per cent reduction in carbon dioxide emissions.

After falling every year from 1991, Britain's emissions jumped by nearly 5 million tonnes of carbon last year, according to the WEC. This was caused partly by an increase in the use of domestic heating fuel during the colder than average winter. Another contributory factor was the United Kingdom's higher than expected economic growth, which accelerated sharply last autumn to between 3.5 and 4.5 per cent per year. The government expects economic growth to remain high this year, falling off to 2.5 per cent in 1998.

Government officials say these latest statistics are entirely within their own forecasts, which predict carbon dioxide emissions will drop by 1 million tonnes between 1995 and 2000.

The British government recently outlined how it planned to reduce carbon dioxide emissions. It includes raising the proportion of electricity from renewable sources to 10 per cent, reducing road traffic and operating a comprehensive household energy efficiency initiative.

Technology programme thinks small

[WASHINGTON] The Clinton administration is planning extensive reforms to its controversial Advanced Technology Program (ATP). These are planned to end years of partisan battles over the programme which provides federal support for civilian technology developments.

William Daley, the Secretary of Commerce, announced last week that ATP would be remodelled to support joint research projects involving two or more companies. It will provide more support for small companies and less for large ones, and will create new links between its projects and the state governments in the regions where they take place.

The wide-ranging reforms are designed to meet criticisms of the programme made by Republicans in the Congress. Since the Republicans won control of the Congress in 1995, ATP has been the main point of disagreement about science and technology policy between the Congress and the Clinton White House.

Many observers believe that ATP would

be more palatable to Republicans if it were to concentrate on small companies and integrate itself with local industrial interests, so that projects would, for example, clearly help batches of auto-component makers in Michigan, or clusters of biotechnology companies in Houston, Texas.

Earlier this year, Lewis Branscomb — a former IBM executive and director of the then National Bureau of Standards, who now heads a Science, Technology and Public Policy programme at Harvard University — produced a report recommending many of the changes now being proposed by Daley.

When President Bill Clinton was first elected in 1992, he pledged to build ATP into a billion-dollar-a-year programme. But Republicans branded it an inappropriate involvement by the government in private-sector activities and sought to eliminate it. As a result the programme has survived on funding of around \$250 million a year, with fierce clashes each year on its funding level.

Colin Macilwain

NASA seeks 'fair exchange' of technical data

[WASHINGTON] The US National Aeronautics and Space Agency (NASA) has launched a campaign to ensure that international research agencies which receive its technical reports at no charge reciprocate by sharing their own published data.

Most do this already, says Terese Ohnsorg of NASA's Science and Technical Information (STI) Office, which handles the exchanges. But some do not, and the agency is reluctant to see this situation continue.

Since the 1960s, NASA has traded technical information — mainly reports and conference proceedings generated by its scientists and contractors — with foreign aerospace agencies, private companies and universities.

Some 90 organizations in 27 countries are on the mailing list to receive the reports. The European Space Agency (ESA) and the Japanese space agency NASDA disseminate the information they receive to hundreds of other organizations. In return, the non-US space agencies are supposed to give their own reports to NASA.

The US space agency periodically looks at these reciprocal agreements to confirm that those who receive technical information are also providing it. "We don't expect that it will be exactly equal, but we expect a good-faith effort," says Ohnsorg.

The current review shows that some recipients have not been contributing their fair share. Ohnsorg plans to write to 15 of the worst offenders this week, asking them to increase their contributions. They could ultimately be removed from the distribution list if they fail to correct the imbalance.

At first ESA appeared to be one of the problem partners. "We realized there were things in the ESA database that weren't getting to us, and we couldn't figure out why that would be," says Ohnsorg.

But this turned out to be a misunderstanding. ESA thought NASA was not interested in certain kinds of information, such as reports on robotics and climate research. When NASA said it was interested, the problem was easily resolved.

That has led NASA to reconsider its guidelines on what kinds of material should be included in the exchanges. With modern computer search capabilities, says Ohnsorg, database managers can afford to err on the side of including rather than excluding information.

NASA's Langley Research Center, which administers the STI database, is forming an advisory group of scientists and engineers to establish guidelines on what kinds of information it should include.

Tony Reichhardt

Canada wins biotech boost of cancer vaccine project

[OTTAWA] Canadian researchers have won out over their French counterparts in a cancer vaccine development project that the Prime Minister, Jean Chrétien, calls "the largest biotechnology investment ever made in Canada".

Joint lobbying by Canadian politicians and scientists has persuaded the French parent company of Pasteur Mérieux Connaught Canada to invest up to C\$350 million (US\$255 million) over 10 years in research to be carried out in Canada rather than France.

The Canadian federal government will invest C\$60 million in the project, which aims to develop vaccines to combat eight forms of cancer that account for 66 per cent of cancer cases in Canada (bladder, lung, prostate, cervical, breast, ovarian, colorectal and melanoma). Repayment of the contribution will depend on sales, projected at as much as C\$10 billion over 10 years.

Canada's lobbying success with Jean-Jacques Bertrand, president and chief executive officer of the parent company, Pasteur Mérieux Connaught (Rhône-Poulenc Group), resulted partly from the country's tax credit system. In Canada, research and development costs companies only 60 cents for every dollar spent, as against about 90 cents in France or the United States.

Unusually, Canada provides no direct subsidies, while in France this type of research could receive subsidies of up to 50

per cent. Canada's C\$60 million investment in this project will be provided through the Technology Partnerships Program, set up by the industry department to create jobs through research in aerospace and biotechnology.

The project could eventually create up to 300 jobs directly and employment for another 250 people in research centres, universities, hospitals and the private sector.

Those who had given their backing to the deal included Canada's three major research grants agencies. The industry minister, John Manley, called the project "a tangible result of this government's strategy for building an innovative economy through partnerships".

About 60 organizations across Canada are likely eventually to be involved. The main focus of the research will be tumour-associated antigens, peptide and carbohydrate technologies, adjuvants, immunomodulators and immunotargeting.

Heather Munroe-Blum, vice-president for research and technology transfer at the University of Toronto, calls the arrangement "a wonderful endorsement of the quality of research in Canada and particularly in the Toronto area."



Manley: 'result of partnership plan'

David Spurgeon

US funds for ITER safe for one more year

[WASHINGTON] United States participation in the International Thermonuclear Experimental Reactor (ITER) project is secure for at least another year after the Clinton administration scotched a move in the Congress to end the collaboration (see *Nature* 388, 110; 1997).

According to administration officials, Federico Peña, the energy secretary, intervened personally last week to secure funding for ITER. Peña apparently persuaded Joseph McDade (Republican, Pennsylvania), chairman of the House of Representatives Energy and Water appropriations subcommittee, which funds energy research, and Vic Fazio (Democrat, California), its senior minority member, that the project should be fully funded.

The House subcommittee subsequently finalized a budget bill that would fund the fusion energy sciences programme at the administration's requested level of \$225 million. The Senate subcommittee went further, increasing fusion funding

to \$240 million.

The bills proposed by both subcommittees would fund non-military science programmes at the Department of Energy at the level proposed by the Clinton administration in February (see *Nature*, 385, 569; 1997). High-energy physics, for example, would get \$675 million — the same as last year — including \$35 million for the US contribution to the European Large Hadron Collider project.

But the Senate bill would provide an additional \$300 million for nuclear weapons research and maintenance, over and above the \$4 billion proposed by the Clinton administration. Senator Pete Domenici (Republican, New Mexico), chair of the Senate panel, said the money was needed to maintain a larger than expected nuclear weapons stockpile. Half of the proposed increase would go to the Sandia and Los Alamos laboratories in Domenici's home state.

Colin Macilwain

Mars rover meets rock with complex past



[LONDON] The Mars Pathfinder mission has scored at least one scientific hit to add to its set of technological achievements: the Sojourner rover has analysed the surface of a rock called Barnacle Bill, and found it to be unexpectedly high in silicates.

Sojourner travelled about 3 metres to the rock on 6 July, extending its alpha proton X-ray spectrometer (APXS), which uses a radioisotope source to bombard the rock surface with alpha particles, and then takes an energy spectrum of the X-rays, protons and alpha particles that return.

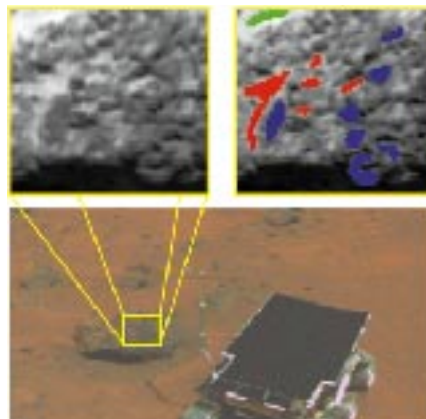
The relative proportions of most of the important elements can be determined in this way. In the case of Barnacle Bill, the measurements imply a silicate content of roughly 58 per cent. This is too high for the most basic category of volcanic rock, basalt, which was expected to be prevalent on Mars's surface.

Barnacle Bill's composition appears to be similar to that of andesite, which on Earth is found above subducted oceanic crust. Either the crust melts, or the mantle melts after the crust has injected water into it; and the melt then erupts from volcanoes to become andesite.

So did Mars once have plate tectonics, similar to those on Earth? If that could be

proved, it would be a considerable discovery because, unless one counts the ice plates of Jupiter's moon Europa, there is no planetary body — apart from the Earth — with evidence for plate tectonics.

Unfortunately, there are other ways to make a rock with this composition. Slow cooling in a large magma chamber, for example, could achieve this, especially in the presence of water. (And a great deal of water is believed to be locked in the rocks of Mars,



Bill's barnacles: pale soil mottles the face of this otherwise dark volcanic rock, highlighting its rough texture. Singled out for optical spectroscopy, the soil looks like oxidized volcanic rock on Earth.

either in the top 10 km of porous crust, or perhaps in the mantle.) Such a chamber could even have been created by the impact of a large comet or asteroid.

More samples would clearly be welcome. On Wednesday 9 July, the rover drove to another rock, 'Yogi'. But a driver's miscalculation meant that it travelled too far, and was left leaning up against the rock, with the APXS at an unusable angle.

By last Saturday, the controllers at NASA's Jet Propulsion Laboratory in Pasadena had succeeded in ordering it to reverse, but only after a few days of confused communication. The first command was thought to have gone astray last Thursday because of a timing error, so it was resent on Friday. Then on Friday evening the lander's computer reset itself and forgot what it was supposed to tell Sojourner. In fact the original command had gone through.

Sojourner should now have had another go at analysing Yogi before heading for a third target, possibly a rock called Casper, whose paleness may mean an even higher silicon content — or just a fine dust coat.

Meanwhile the lander is collecting weather information and preparing a colour panorama of the area. Up-to-date information can be found at <http://mpfwww.jpl.nasa.gov> and at many other Web sites around the world.

Stephen Battersby

NASA 'must fill gaps' to finalize streamlined plans for human expedition

[WASHINGTON] Planners with the US National Aeronautics and Space Administration (NASA) have come up with a 'reference mission' for a human expedition to Mars that would, they suggest, cost about one-tenth the amount of earlier proposed missions.

But the plan is still rough, and there is as yet no political or financial commitment to the project. The agency also lacks some of the technical tools and basic knowledge needed to send people to Mars, according to a report published by the National Academy of Sciences last week.

Even before this month's Pathfinder landing, which has generated more public interest than any other recent space mission, NASA's administrator, Daniel Goldin, had challenged a team of engineers at the Johnson Space Center in Houston, Texas, to find a way of sending humans to Mars in less than a decade for \$25 billion.

That sum would roughly match the US investment in the International Space Station. A Mars expedition would be expected to launch well after the station is completed, probably between 2010 and 2020.

The Johnson study team has not yet met Goldin's cost target. But it has come within a factor of two, says Lewis Peach, director of advanced projects for NASA's Office of Space Flight. This compares with NASA estimates of the late 1980s, which priced a more complex Mars exploration programme at \$400 billion.

The current plan would send a crew of six to stay on the surface of Mars for 500 days. Technical innovations that have lowered the cost include inflatable habitats for the crew and the use of in-situ propellant — extracting oxygen from the planet's atmosphere to make fuel for the trip home, instead of carrying it on the outward

journey to the planet.

Both advances reduce the amount of material launched from Earth. The plan also assumes new economies in future launchers used to reach Earth orbit. The Johnson team briefed Goldin last week on the technological requirements for a human expedition.

NASA is focused on sending robotic spacecraft to Mars at every 26-month launch opportunity between 1998 and 2005. Science has priority on these flights, which will land on the planet in several locations, survey it from orbit, and return rock samples to Earth.

But technology to support future human expeditions will also be developed on the science missions, says Peach. For example, if a prototype fuel-production plant works as advertised on the 2001 mission, a similar device flown in 2003 could produce enough fuel to launch a

small sounding rocket from the Martian surface carrying scientific experiments.

Not all the hurdles facing a manned expedition relate to hardware. An academy panel warned this week that NASA should be investing more money and attention on the problem of human survival in space if it wants to reach Mars by 2010.

Areas singled out by the panel as needing technological improvement include life support systems, such as waste recycling and food production; environmental monitoring on a Mars ship; space suits for exploring the Martian surface; and human factors such as crew training and scheduling.

Another academy committee said last December that the space agency would also have to improve its understanding of the biological effects of radiation before sending astronauts to Mars.

Tony Reichhardt

Japan's life sciences take integrated road

[TOKYO] Japan's main science policy-making body is expected to approve a new ten-year plan for promoting the life sciences later this month. The plan, by the Council for Science and Technology, puts particular emphasis on strengthening areas of research with possible medical applications.

The final draft of the plan, released on the Science and Technology Agency's website, calls for an approach that integrates the study of human genetics, brain science, cancer and developmental biology. It claims that these should focus not only on the molecular and cellular level, but also on the whole organism.

It also seeks more research into cancer, genetic diseases and diseases of the brain — reflecting the fact that Japan faces a steep increase in the number of old people early next century.

This is the twenty-fourth in a series of reports issued by the council, which is attached to the office of the prime minister but located in the Science and Technology Agency, since its foundation in 1959. The expert group responsible for drafting the document was led by Yoshitaka Nagai, director of the Mitsubishi Kasei Institute of

Life Sciences south of Tokyo. He points out that genetic diseases provide a good example of the proposed integrated approach, as they demonstrate that the link between genotype and phenotype is not always straightforward, and that such diseases can seldom be fully understood through studies at the molecular or cellular level alone.

The report says Japan could open up a new economic frontier by developing medical, food and information industries based on life science research, as well as new electronic products such as biosensors and neurochips.

But it acknowledges many problems in Japan's present research environment — for example, Japan's comparatively low input of genome data to its databases. (The report notes that only about 10 per cent of the genome data at the DNA Database of Japan in Mishima is of Japanese origin and calls for greater output of such data by Japan.) It also highlights its lack of laboratory animals, in particular primates, for modelling human disease and testing new medical techniques.

The focus on biomedical research appears to mark a clear departure from the

central role long given to molecular biology in the life sciences in Japan. According to Nagai, the report's conclusions are essential if humans are to be placed at the centre of life science research. "Reductionism has been an immensely powerful tool in biological research, but it is becoming clear that integrated approaches are needed at the moment," he says.

The report points out that the US National Institutes of Health alone spend about ¥1,000 billion (US\$8.8 billion) each year on life science research. It calls for an expansion of Japan's life science budget over the next ten years, but makes no specific financial recommendations.

Indeed, in the present climate of fiscal restraint, further large expansion of the life science budget seems unlikely, given that the government has already promised a marked increase in the budget for brain science (see, for example, *Nature* 385, 104; 1997). But, according to Kanji Fujiki, director of the office of life science promotion at the Science and Technology Agency, the report may help to "make more efficient use of existing resources". **Robert Triendl & David Swinbanks**

Clinton backs Congressional efforts on genetic discrimination

[SAN FRANCISCO] US President Bill Clinton announced on Monday that he plans to propose legislation to prevent health care companies from discriminating against consumers on the basis of genetic information. He said his bill would strengthen other legislative proposals, partly by adding sections that would not allow disclosure of genetic information unless the Secretary of Health and Human Services determined that this might support biomedical research.

Pledging his commitment to non-discrimination legislation, Clinton released an 11-page report from the Department of Health and Human Services. This concludes that current federal protective measures contain significant weaknesses concerning the denial of coverage by health plans of individuals, the setting of premiums, and the disclosure of information.

Also, although 19 states have protective measures against the misuse of genetic information by health insurers, most do not define such information sufficiently broadly to include family history, medical records and physical exams. And self-insured employers are exempt from such laws.

Unless laws specifically address the problem of misuse of genetic information, individuals will be afraid to obtain information that could be vital to their lives, the president said at a press conference. He was accompanied by Secretary of Health and Human Ser-

vices Donna Shalala, who had flown in from Chicago fresh from delivering a speech on genetic discrimination to a convention held by Hadassah, a Zionist women's organization that emphasizes education and health care, and by Vice President Al Gore.

Clinton urged Congress to move quickly on his legislation, which would build on HR 306, a bill introduced by Congresswoman Louise Slaughter (Democrat, New York) to prohibit health plans from denying, refusing to renew, cancelling or changing the terms of a health insurance policy based on genetic information. Health plans would not be able to require genetic tests or to disclose genetic information to a third party.

The President's bill is intended to extend genetic information protective measures recently enacted under the Kassebaum-Kennedy health reform package to self-insured employers, uninsured workers, people switching jobs, and other groups. The Kassebaum-Kennedy law prohibits insurance companies from refusing health coverage based on a person's medical history, including genetic information. HR 306 also applies tax penalties to violators and allows individuals to sue their health plan for punitive damages if they feel that they have been discriminated against.

Members of Hadassah, the largest women's organization in the United States with 300,000 members, called such legisla-

tion vital. "There's been a clamouring for a commercially available test in our community, but when we started to become aware of the history of insurance discrimination on this issue, it had a chilling effect," says Amy Rutkin, director of American affairs for Hadassah. The group has recruited co-sponsors for the bill and has urged its members to discuss their concern about discrimination in their communities.

HR 306 has 135 co-sponsors among legislators and 65 endorsing organizations, but has so far failed to make the commerce committee agenda. Olympia Snowe (Republican, Maine), is co-sponsoring the bill in the Senate, which also has been slow to move on it. One reason is that biotechnology and pharmaceutical industry executives are concerned that such legislation would excessively protect health databases from research usages and distinguish genetic information from other medical data.

Some, such as Paul Billings, chief medical officer at the Heart of Texas Veterans Integrated Service Network and an expert in genetic discrimination issues, see Clinton's statement as a watershed moment in US history by declaring that genetic discrimination in health matters is unlawful.

"We are recognizing how important an environment of non-discrimination is to maximizing the benefit of this technology," says Billings. **Sally Lehrman**

'USA should fund half transatlantic links'

[MUNICH & WASHINGTON] The United States should pay half the costs of the circuits linking its research networks to those in other continents, according to a paper published last month by Renater, the French research network organization.

The United States does not at present contribute at all to links with Canada and Japan, and it pay less than 10 per cent of the costs of transatlantic links with Europe.

The debate about who pays for intercontinental links has become heated in recent years because of the growing costs. The estimated costs of the Europe-US links, whose capacity of 270 megabits per second (m.b.p.s.) is expected to double in the next two years, are around \$50 million a year.

The disparity has arisen for two reasons: the history of the Internet, and the decision of the US National Science Foundation in 1995 to shut down its own network, NSFNET (see *Nature* 387, 8; 1997).

A decade ago, universities and research institutes outside the United States were happy to pay the full cost of intercontinental connections to gain access to scientific information on Internet-based US research networks, as they had relatively little to offer the US research community in return.

Having become accustomed to such courtship, the United States is proving unwilling to help to pay for connections now that the balance of potential information exchange has evened out. National research networks, many operating with the same level of broadband capacity that the United States now uses, have been developed in many countries outside the United States.

Whereas research agencies in many countries provide a special network backbone for use by universities, such as JANET in the United Kingdom, the NSF in the United States decided to stop doing this in 1995. Encouraged by pro-market sentiment in the Congress, the agency shut NSFNET and told universities to use one of several

commercial networks instead.

But, since that decision, an unexpectedly sharp growth in commercial Internet traffic has resulted in severe congestion, and researchers with large data-transfer requirements at US universities are scrambling to join the experimental, high-speed 'Internet 2' network being developed by the NSF (see *Nature* 380, 377-381; 1996).

US officials continue to insist that they will not subsidize international Internet links when they do not subsidize domestic ones. Steven Goldstein of the NSF's networking and communications division says the agency has a budget of \$50 million for research and infrastructure, and its advisory board has suggested that 10 per cent of this should be spent on international links.

In 1995, the G7 group of industrialized countries approved an initiative introduced by US Vice-President Al Gore to develop an integrated worldwide information infrastructure. As part of this initiative, a working group called the Global Interconnection of Broadband Networks (GIBN) was set up.

At its meeting in Tokyo in January, GIBN, which comprises representative experts from all G7 countries, agreed that it was necessary to establish an international infrastructure for broadband applications and other requirements of science and education, but that a fairer way of sharing the costs of intercontinental links should be found. France agreed to develop a model for cost-sharing, based on links between Europe and the United States, through Renater.

The model concludes that the user requirement for transatlantic access in Europe and the United States is roughly equal, and that payment should therefore be shared equally. Because telecommunications costs are much higher in Europe than in the United States, the model allocates cost-sharing on the basis of half-circuits, with either side financing its own half.

The model also assumes the rationaliza-



tion of the fragmented capacity in Europe which is spread across 10 links, in the expectation of the establishment of a single interconnection point on either side of the Atlantic. It will be discussed at the next GIBN meeting in October.

It has already received favourable responses from European research networks including the DFN, the German research network, which launched its own 90 m.b.p.s. transatlantic link this year. The full DM20 million (\$11.4 million) annual costs of this link are met by German universities and research institutes, which causes some resentment at the DFN.

In May the NSF issued a call for proposals to support interconnections between the United States and other countries, with an annual budget of \$4.5 million. It will spend another \$0.5 million on a single connection point, in Chicago, through which international users will be able to access Internet 2.

Dai Davies, general manager of Dante, the company in Cambridge, England, that organizes international network services for the European research community, describes the budget as "insultingly small".

Goldstein says that the United States does not yet have a view on the French model, but he holds out little hope for a larger US contribution to international networking costs. "We only have a fixed amount of money, and we don't provide commodity networking for our own universities," he says. "We understand the Europeans are angry, but there isn't a lot we can do about it."

The first intercontinental supercomputer link using high-speed telecommunications networks was established last month between two Cray T3E supercomputers at the University of Stuttgart and the Pittsburgh Supercomputing Center. This is the first international link using the very-high-speed Backbone Network Service (vBNS), which connects all US supercomputers. The transatlantic connection is provided on an experimental basis at no cost by the Canadian company Teleglobe.

Alison Abbott & Colin Macilwain

Encryption technology divides policy-makers

[LONDON] The United States clashed with European Union (EU) countries last week over government controls on encryption technology. Their ministers were meeting in Bonn, Germany, to set common standards for communication networks such as the Internet.

Despite signing a joint statement with the EU supporting 'free choice' in the sale and use of

encryption products, US officials maintained that they had no intention of relaxing US controls banning the export of sophisticated encryption products.

The US commerce secretary, William Daley, said he understood that encryption was needed to prevent eavesdropping during the electronic transmission of credit card numbers and sensitive

documents such as contracts. But he warned that the technology permitted should not be so sophisticated as to allow terrorists and criminals to hide their activities from law enforcement agencies.

EU ministers, as well as business leaders in the United States and Europe, believe that the restrictions inhibit trade in encryption technology. □

NIH urged to address chimp care 'crisis'

[WASHINGTON] A \$7 million-a-year Chimpanzee Management Program — or ChiMP — should be established at the office of the director of the National Institutes of Health (NIH) to provide lifelong care for 1,000 research chimps in the United States. So concludes a report from the National Academy of Sciences published this week.

Dani Bolognesi, director of the Duke Center for AIDS Research in North Carolina, and chair of the panel that produced the report, says that central control would deal with a crisis in the management of the chimpanzees, many of which are no longer needed for research. The cost of the programme would be similar to the costs at present borne by various government agencies responsible for the chimps, Bolognesi says, and should not come out of the NIH biomedical research budget.

The report rejects the culling of the chimps. But a minority report by panel member Sarah Williams-Blangero, of the Southwest Foundation for Biomedical Research at San Antonio, Texas, says that culling would maximize the quality of life of the remaining population, and should be considered.

The study also rejects demands from animal rights groups that the government should stop using the chimps for research, and should help to pay for sanctuaries where they could retire. Bolognesi says that chimps managed under the new programme could retire later to such sanctuaries if and when they are established. The panel proposes a

four-year moratorium on chimp breeding, to be reviewed annually.

Chimpanzee care is a hot issue in the United States because there is a surplus of the animals, worsened in recent years by an NIH-sponsored breeding programme. They are expensive to care for, and the public will not accept that they be neglected or killed. The normal life span of a chimpanzee is 40–45 years.

The 1,000 or so chimps owned and supported by government agencies — including the National Cancer Institute, the Centers for Disease Control and the US Air Force — are kept at six centres across the country, while another 500 are in private facilities.

Some of the chimps have AIDS or hepatitis and are kept in solitary confinement. But Bolognesi says that their overall plight is not as bad as he had feared at the outset of the study: "There are a few problems but by and large they are fairly comfortable," he says. But, as costs go up and reimbursement from the federal government goes down, "the facilities are going to go bankrupt", he says. "They are trying to get rid of the chimps as fast as they can."

Bolognesi predicts that good, stable management by ChiMP could revive flagging research interest in the animals, lifting the threat that anyone who makes use of the animals will incur lifetime care costs.

The report is unlikely to mollify animal activist groups, which have demanded that NIH set aside up to \$100 million to help pay for retirement sanctuaries for the chim-



Short sighted: sponsored breeding programmes have created a surplus of research chimpanzees.

panzees (see *Nature* 381, 722; 1996). The NIH may be reluctant to accept responsibility for all the chimps — especially the director's office, which has been seeking to pare down its functions.

And some observers question whether Congress will support a programme intended expressly to look after chimps. "It would be far more compelling if we attached this to some human benefit rather than simply establishing a chimp welfare program," says Joe Erwin, past president of the American Society of Primatologists.

Some scientists are concerned about the lack of logic in society treating chimps better than it does other research animals — or disadvantaged humans, for that matter. "We can't tell you that the chimpanzee is the only animal that should be accorded this privilege," says Bolognesi. "But we do have a crisis with the chimps right now."

The academy report says that the ChiMP programme would operate under the supervision of an independent board of scientists and other interested parties. It says that its proposals could win the approval of scientists, the public and members of Congress, but that "NIH must work hard to achieve that breadth of approval".

Criticism of the care of chimpanzees in the United States has focused on the treatment of 600 or so animals that are held by the Coulston Foundation in New Mexico, of which 200 are paid for by the government. An official at the academy says that the study had received "very little information" about the animals held by Coulston, which has been lambasted by animal rights groups for its standards of care.

The US Air Force has been trying to dispose of its 135 chimps for many years. But the budget system makes it difficult to transfer money from the air force to, for example, the NIH for the future care of the animals.

Colin Macilwain

Small increase planned in German budget

[MUNICH] Jürgen Rüttgers, Germany's minister for education and research, may be about to resurrect his reputation as 'minister for the future'. Last week, the German cabinet approved a funding increase of just under 1 per cent for education and research next year — almost twice the increase for the budget as a whole.

The research budget will now total nearly DM15 billion (US\$8.5 billion). Rüttgers says that, if the funding increase is approved by parliament, the extra money will be allocated to previously established research priorities, including biotechnology, molecular medicine, information technology and environmental technology.

He will keep promises to maintain the level of special funding to support the development of research infrastructure in east Germany, and to increase by 5 per cent the budgets of the Max Planck Society and the Deutsche Forschungsgemeinschaft, Germany's university grants council. The budget of the Fraunhofer Society, which

runs 47 applied research institutes, will rise by 3 per cent.

Because the relatively small increase in budget will not cover all these plans, Rüttgers has also proposed cuts. Most significant is his decision to reduce spending on nuclear safety, despite Germany's proximity to reactors in central and eastern Europe whose safety is uncertain.

Under the budget proposal, funding for safety research for nuclear power stations would be cut by 3.6 per cent, and for decommissioning nuclear power stations by around 6 per cent.

Rüttgers is proposing to cut by 11 per cent Germany's contributions to the Vienna-based International Atomic Energy Agency. This will probably affect the agency's voluntary technical assistance fund, which supports a wide range of research, from reactor safety technology to the medical consequences of radiation exposure. The combined cuts will release just over DM50 million.

Alison Abbott

Malaria meeting charts rocky path ahead

[SCHEVENINGEN, THE NETHERLANDS] US proposals to establish a common, centrally managed fund for malaria research were firmly rejected at an international meeting in Scheveningen last week. But a positive outcome of the meeting was a decision on the next steps to take in combating malaria.

The high-level meeting was a follow-up to an international meeting on malaria research held in Dakar, Senegal, in January, where the world's research agencies, charities and major donors met for the first time to explore ways forward (see *Nature* 386, 535; 1997). The movement has since been named the Multilateral Initiative on Malaria (MIM).

The meeting resulted in a clarification of MIM's *raison d'être*, with a shift in focus away from relatively narrow issues of research management towards the wider strategic goals of obtaining an increase in funds for malaria research, and pushing the disease up the international political agenda. This shift was reflected in the decision to replace MIM's ill-defined collegial structure with a more formal organization and to set up a series of working parties.

The degree of attention now being focused on malaria was reflected in the calibre of the participants at the meeting. The US delegation alone included Harold Varmus, the director of the National Institutes of Health (NIH) and Anthony Fauci, director of the US National Institute of Allergy and Infectious Diseases.

Coordination, not pooling

Also present were Fotis Kafatos, director of the European Molecular Biology Laboratory in Heidelberg, George Radda, director of the UK Medical Research Council, Robert Howells of Britain's Wellcome Trust, and senior officials from the World Bank, the European Commission (EC), the World Health Organization (WHO) and the Organization for African Unity. Major pharmaceutical companies were also represented for the first time.

The US proposal came under intense criticism from all sides, with opponents calling it premature and unworkable. An alternative proposal was equally rejected, which, while creating a common application and review process, would still have allowed individual agencies to decide whether to fund projects approved by a common review body. Mark de Bruycker, an EC official, pointed out that, as the commission is legally obliged to peer-review all research proposals, it would be unable to take part in such a scheme.

Echoing comments by other agencies, he argued that coordination could be achieved more simply by agreeing on specific priorities, and then inviting researchers to apply for funding for these through existing

agencies. Howells agreed that "pooling resources is not desirable". Others argued that malaria should be considered as part of the overall collaboration between agencies on issues of health in developing countries, and that setting it apart would weaken inter-agency collaboration. But Varmus dismissed such positions as defending "business as usual, with a little more enthusiasm".

Letters of interest

Many at the meeting, while encouraged by Varmus's prominent lead in promoting malaria research, were concerned that US hyperactivity could upset established working relationships between the agencies involved in malaria research. Indeed, apprehension at US calls for radical change was fuelled by the fact that its interest in health issues in Africa is relatively recent, whereas Europe — the United Kingdom and France in particular — has a long tradition of supporting research on the continent.

"I can understand Varmus's impatience, but at the same time he must respect the fact that actually Europe has delivered here and the US has not," said one senior official from a European funding agency.

The root of the row was a perhaps overzealous decision at the Dakar meeting to issue a call for 'letters of interest' in malaria research. The organizers originally intended this only to yield a rough idea of the needs of researchers. But many scientists interpreted it as a formal call for proposals that would be followed up by funding.

The meeting found itself in the embarrassing position of having 138 letters of interest requiring new funding of millions of dollars, whereas MIM had not even begun to address the fundamental problem of how to win an increase in funds.

In putting the cart before the horse in this way, the organizers triggered what many agreed was a premature debate over how any research programmes should be managed, which took the meeting to the brink of a fiasco. At the same time, the mini-crisis prompted a rethink of MIM's purpose. Several delegates reminded the meeting that MIM's efforts would come to nothing unless it focused on the fundamental issue of boosting the paltry funds now available for malaria research (see *Nature* 386, 535; 1997).

"Talking about research networks is not going to put malaria on the global agenda," said C. Ok Pannenberg, a senior official at the World Bank. One funding agency official said that if funders were to invest they would need to hear consensus on such strategic questions as where most money should go — in vaccines, drugs or basic research — and whether more field sites should be set up in Africa, and, if so, how many.



Varmus: plans for a centrally managed fund for malaria research failed to win universal support.

As a result, a core organizing committee, which includes the Wellcome Trust, NIH, France's Institut Pasteur, the EC and the Organization for African Unity, has been created to address such issues.

Winning funds for research

The outcome of the meeting was ultimately "extremely positive", said a funding agency official, because it "put the discussion on the next level up", enabling MIM to start 'politicizing' malaria and getting more resources. Howell added: "There is now a much clearer picture that MIM is there to attract funding for malaria research."

A series of working groups was also set up, with WHO's tropical research division accepting responsibility for preparing a compendium of the mandates, procedures and research carried out by the various agencies, with a view to facilitating collaborative research proposals. NIH is to report on the major basic research challenges, while the World Bank will prepare a macroeconomic analysis of malaria-related issues.

Responsibility for public relations, which emerged as a priority, was allocated to the Malaria Foundation, an independent charity based in Washington. It will also develop a plan for improving Internet access in Africa, working with the US National Library of Medicine, the EC and the World Bank.

This approach was endorsed by Pannenberg, who argued that MIM must realize that progress in fighting malaria can be achieved only by "a long-term concerted effort by a powerful group of players", but with the various agencies working "in parallel".

Meanwhile, the very existence of an influential lobby for malaria research is already paying dividends, according to one official. "Everything that was going on here [at The Hague] helps us to find ways to get money into malaria," he said after the meeting. "It empowers me to say to my colleagues, as I did, that 'I think something very good seems to be happening here'; I wouldn't do that if I thought this was no good".

Declan Butler

US government forbids export of equipment to Indian nuclear centres

[NEW DELHI] The Clinton administration has banned US companies from exporting equipment to three Indian nuclear facilities, following reports that they are "involved in developing weapons of mass destruction".

But India has denied the charges. A government minister said the ban was unlikely to affect continuing activities, and some scientists have described it as a blessing in disguise, as it will encourage India to develop its own technologies.

The curbs apply to the Bhabha Atomic Research Centre in Mumbai (formerly Bombay), the Indira Gandhi Centre for Atomic Research in Kalpakkam near Chennai (formerly Madras) and the Indian Rare Earths Centre at Alwaye in Kerala state.

The Indian government has been told that similar export curbs have been applied to institutions in Pakistan, Israel and Russia.

University of California sues Genentech

[SAN FRANCISCO] The University of California is suing the biotechnology company Genentech, alleging that the company is violating the university's patent on

recombinant DNA vectors in its process for making human growth hormone products. The suit, filed last Thursday in the district court in San Francisco, challenges Genentech over its production of Nutropin, a genetically engineered growth hormone in an injectable liquid form approved in 1994.

Genentech also makes a similar product called Protropin, which is sold as a powder and was biotechnology's first successful product launch in 1985. The University of California has also disputed Genentech's right to make that product without a licence to its transfer vector. That suit, filed in 1990, will be addressed in a US district court in San Francisco on Friday (18 July).

Damages sought over radioactive material

[WASHINGTON] Lawyers acting for a pregnant scientist who ingested radioactive material at the US National Institutes of Health (NIH) in 1995 are again threatening to sue the government for negligence. They are seeking "significant damages" for their client, Maryann Ma, and her husband and co-researcher, Bill Zheng.

In a petition to the Nuclear Regulatory Commission, which oversees the use of radioactive isotopes at NIH and other government laboratories, the lawyers charge that NIH "intentionally underestimated" the

radiation dose received by Ma when she ingested phosphorus-32 (see *Nature* 377, 568; 1995). They say that NIH has continued to violate federal regulations on the handling of such isotopes since the incident.

Former research chemist tipped for Commons role

[LONDON] Michael Clark, the Conservative Member of Parliament for Rochford and a former industrial research chemist, is expected to be named as the new chairman of the House of Commons Select Committee on Science and Technology. The science committee is one of only three committees to have been allocated a Conservative chairman, following the party's rout in the general election in May; the allocation of such posts traditionally reflects voting strengths within Parliament. Clark has been actively involved in a number of technology-related select committees in recent years, and is also a former honorary secretary of the Parliamentary and Scientific Committee.

Spanish geophysicist nominated to run ESF

[LONDON] Enric Banda, a distinguished geophysicist and former secretary of state for universities and research in Spain, has been recommended as the next secretary general

of the European Science Foundation. He would succeed Peter Fricker, whose term of office ends next May. Banda's recommendation will go for approval at the next meeting of the foundation's executive council in September, and to the general assembly in November for ratification.

Foundation president Sir Dai Rees said there had been a strong field of applicants, and expressed confidence that, under Banda, the organization will continue to play an active role in supporting European-level research.

US mayors ask Congress to increase science cash

[WASHINGTON] The mayors of Pittsburgh, San Jose, Seattle and a dozen other major US cities have sent a letter to Congress backing increased spending on scientific research and education. The mayors say that science will support industrial development and jobs in their cities. "The industries that we foster in our cities consistently identify the research and trained students that result from the federal support of science as their most vital resource," says the letter.

Housing and other pressing urban needs compete with science for money in the congressional budget process. The Science Coalition — the lobbying group that organized the mayors' letter — hopes that the message will show that science is a priority

for urban America. But the mayors of the largest cities, New York, Chicago and Los Angeles, did not sign the letter.

Canada dismantles food and drug posts

[MONTREAL] The posts of 191 researchers and others responsible for ensuring the safety of food and drugs sold in Canada are being eliminated by the federal government. More than 70 researchers in the food directorate have already protested in a petition, saying the cuts will "seriously affect the future health of Canadian infants, children and adults". Work formerly done in the drug research bureau will now be carried out by pharmaceutical companies submitting drugs for approval, by university researchers — many of whom are funded by pharmaceutical manufacturers — and by regulatory agencies of other countries, including Britain.

British science lectures head for Japan

[LONDON] Britain's Royal Institution has reached an agreement to present in Japan an edited version of last year's traditional Christmas lectures, which were given by Simon Conway Morris of the department of Earth sciences at the University of Cambridge, and entitled "The History in our Bones".

The lectures, which attracted a record television audience in the United Kingdom, will be broadcast by the national Japanese TV network, NHK. They provide a perspective on the various forms of life on Earth, and on the evolutionary process that culminated in the superiority of *Homo sapiens*. The cost of the broadcasts will be covered by a sponsorship deal involving several major Japanese companies, including with the Tokyo daily newspaper, *Yomiuri Shimbun*.

Israeli science minister will serve one year

[JERUSALEM] Israel has a new science minister — Michael Eitan, who has served as chairman of the coalition factions in the Knesset for the past year. Prime Minister Benjamin Netanyahu has held the science portfolio himself since the resignation of Ze'ev Binyamin Begin late last year. But the new minister will serve for only a year. Then he will exchange posts with Silvan Shalom, appointed deputy defence minister.

The science ministry's director-general, Zvi Yanai, announced on the day Eitan's appointment was approved that he would resign as soon as Eitan finds a replacement. But Yanai, who was appointed three-and-a-half years ago under the previous Labor government, said that his resignation was not linked to Eitan's appointment.

Limit the number of grants per person

Sir— In these times of tight funding, it is surprising that some Principal Investigators (PIs) at the US National Institutes of Health (NIH) have seven or more research grants. (In a recent casual search of the NIH database, I found one investigator with 12 grants.) This distribution has not undergone major changes over time (see *Nature* **363**, 578; 1993).

In general, the more grants one has, the more each is worth. Rather than the infrastructure of one grant assisting another, the opposite appears true. The science done in big laboratories may be of good quality but, for every additional grant given to a big laboratory, several independent investigators are given no chance.

In 1996, three investigators had seven research grants, each averaging almost \$900,000. For the cost of funding these three investigators, almost 80 PIs were unfunded. If NIH were to limit the number of research grants to two per PI, approximately 3,000 new RO1 grants for young investigators could be funded with no change in the NIH budget and without eliminating any currently funded laboratories.

An examination of the Nobel prizes in

biology and medicine makes it clear that the work for which the prize was awarded was done while the investigators were not holders of multiple grants. Nobel prizewinners are obviously bright, well funded and have excellent students, but they don't get more Nobel prizes. Can one buy creativity in science? In the competitive field of molecular biology, the average number of NIH grants per cited author (of the top 10) is 2.1. Apparently more NIH funding is associated with less interesting results.

Grant collectors deprive young people of independent research opportunities and also keep them out of academic institutions. If an applicant doesn't have a continuing line of NIH funding, tenure approval is unlikely. Much discussion has been devoted to bringing young investigators, and particularly minorities, into science. But how can a newcomer compete with established scientists with many grants and dozens of students and technicians?

The NIH should examine, by some objective measure, whether channelling vast sums into big laboratories does produce more productivity per dollar than grants to smaller laboratories. If it doesn't, then NIH needs to revise the rules by which

grants are awarded. One possibility would be to limit the number of grants per PI. About 1,200 PIs (5 per cent of the total grant recipients) would be affected if awards were limited to two per PI.

If limits were placed on the number of grants per PI, the biggest laboratories would get smaller, and more junior faculty would become independent PIs and generate the key ideas that determine the future path of science. Also, on obtaining a maximal number of grants, PIs would be relieved of the demand (self-inflicted and from administrators) for more proposals.

There are obviously many ways to set limits on grant support and none is immune from abuse and error. But we, the research community, should tell NIH, Congress and the American public that we are willing personally to sacrifice funding opportunities to obtain a more equitable distribution. If we are then rewarded with increased funding by Congress, the increase will not be squandered.

Frederick Sachs

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True to Tony Blair

Sir— Thank you for your coverage of my appointment as parliamentary private secretary to the science minister, John Battle (*Nature* **387**, 745; 1997). There is, however, one inaccuracy that I hope you will allow me to correct.

I enthusiastically supported Tony Blair in the leadership election three years ago. In fact I nominated him and worked actively in the campaign team to get him elected. There is no truth in your assertion that I declined to vote for him.

Anne Campbell

*(Member of Parliament for Cambridge)
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Getting ahead of one's self

Sir— In these days of excitement about the Beppo-SAX identification of X-rays associated with gamma-ray bursts and the subsequent observations of possible optical counterparts, a frantic atmosphere has grown among astronomers

anxious to establish priorities.

I noticed with surprise the Letter by Sahu *et al.* (*Nature* **387**, 476–478; 1997) which appears to have been received on 31 March and starts by explaining that: "The optical counterpart of GRB970228 was observed with the HST Wide Field and Planetary Camera (WFPC2) on 26 March and 7 April..."

I doubt that this is a misprint, because a NASA press release (97-63) of 1 April announces that the same authors have submitted a paper to *Nature*.

I suggest that you publish a correction asking readers to insert in the above-mentioned article, after the title, the sentence "Note added in proof".

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● **The original submission was based only on the Hubble data of 26 March. A revised version containing the 7 April Hubble data was the one sent to referees and published. It is Nature's policy in such circumstances to change the received date, but by an oversight we failed to do so on this occasion.**

— Editor, *Nature*.

Capital Letters

Sir— I write on behalf of 27 scientists in the School of Biological Sciences at Flinders University who have signed this letter.

Despite the widely held view that Letters to *Nature* present results of exceptionally high scientific achievement and outstanding originality, the Australian federal government's Department of Employment, Education, Training and Youth Affairs has recently decided that Letters to *Nature* are not an appropriate measure of scientific output for the purposes of determining infrastructure grants to Australian universities¹.

James Watson and Francis Crick might be interested to learn that they have perhaps been found wanting by modern Canberra bureaucrats. *Nature* itself may also feel diminished by this decision.

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¹ 1997 Higher Education Financial and Publications Research Data Collection Specifications for Preparing Returns (Dept of Employment, Education, Training and Youth Affairs, Canberra).

Need for change in Japan's universities

Japan's universities need strong leadership to give young scientists, foreign staff and women the opportunities that are denied them. Academics should have more of a sense of crisis over what is an unacceptable situation.

Yoshinori Kumazawa

As a faculty member of a Japanese national university, I feel shamed by the justifiable criticisms that have been made of our national universities over their discrimination against foreigners seeking tenure status (see, for example, *Nature* 383, 651 & 654; 1996). It is frustrating that university academics and officials are unable to remedy this and other serious problems that exist throughout Japan's university system.

Foreign staff in Japanese universities are essentially expected to have only supplementary, educational roles in subjects in which Japanese faculty members are not as competent — teaching foreign languages, for example. Why are the Japanese so reluctant to accept foreign faculty members on equal terms? I believe that the answer lies in protectionism and conservatism among some Japanese scientists, university officials and politicians. They are afraid, consciously or unconsciously, that unrestricted granting of tenure to foreigners might lessen the chances for Japanese nationals. They are caught up in the negative aspects, and do not realize that the acceptance of more foreigners on an equal footing could revitalize Japanese academic institutions.

Lack of openness

I was a postdoctoral fellow in the Department of Molecular and Cell Biology, University of California at Berkeley, from 1990 to 1993. I found the educational and research system of the Berkeley campus to be very different from that of Japanese universities. Faculty members were from all over the world and of different ethnic origin, nationality and sex: everyone was evaluated by a common scientific standard. There is little chance of the Berkeley style being introduced to Japan, even in part, as I do not think that the national universities are highly conscious of the need to change the balance of the nationality or sex of tenured staff.

To take sex discrimination, there is only a small proportion of female faculty members in Japanese national universities (for example, approximately 3 per cent in the graduate school of science in my own university, and no females among 25 faculty members in my department). I once pointed out in an employment committee meeting that one-sixth of the students in our department are female, and asked how we could give them hope for an academic career. Many of my fellow committee members were surprised at my question and one (now retired) told me

that women are poor at walking in the field and thus cannot become good geologists. Some male scientists attribute the scarcity of female faculty members simply to a lack of promising scientists in the female population. I would like to ask them whether they think young females have been given enough chance to develop into established scientists.

Japanese national universities have also been criticized for discrimination against young scientists of both sexes. Only a small number of vacancies for faculty positions are advertised, not because the number of positions is small, but because almost all positions are automatically tenured and many vacancies are filled by people acquainted with influential members of the department.

Even if a young scientist is lucky, and does enter the academic heaven of tenure, will he or she then have a pleasant career? I believe not. The main Japanese universities, especially their chemistry and biological science departments, operate the notorious *koza* system. Many, though not all, professors of *koza* do not expect associate and assistant (*joshu*) professors to carry out research or publish independently. If these people were to conduct research that did not interest their professor, they might find themselves facing various obstacles.

One way in which the associates and assistants adapt to this environment is to carry out the professor's research plans faithfully (to act as postdoctoral researchers or supervisors) and to produce many papers with the professor as an honorary author. They thus hope to become full professors as soon as possible, and then repeat the process with their own young protégés. It is very difficult to survive in this environment without losing one's scientific independence. Recent efforts to change the *koza* system seem to me to have had no substantial effect.

Japan's universities have a huge organization but no strong leadership. In the United States, institutions compete for excellent faculty members and students as this leads to more grants and financial donations. On the other hand, Japanese national universities automatically receive a certain minimum

level of their funding from national taxation. The public remains largely ignorant of their plans and has little or no say in them.

The infrastructure of Japanese universities tends to be of far lower quality than that of European and US universities, but, in my experience, Japanese universities are more likely to be equipped with modern, expensive machines (for example, ultracentrifuges and DNA sequencers). Professors in major national universities sometimes obtain large amounts of money that cannot be used to employ researchers, so they buy expensive machines, often duplicated in individual *koza* at the same institution. This is not only a waste of money and space, but can inhibit young scientists from becoming independent. Funds for expensive equipment could often be better used to foster creative talent via, for example, postdoctoral fellowships.

Source of leadership?

Where can Japan find the leadership to address these problems? Can the universities reform themselves without outside interference? As things stand, because some university faculty members lost their jobs during the Second World War as a result of their opposition to the government, all policy decisions are made democratically by the relevant people in the universities independently of the government. Under this system, any radical reforming proposal is likely to be opposed by those faculty members who consider that change will not bring them benefit. Even the president of a university cannot make a decision on such a matter without first obtaining comprehensive consensus.

I fear that substantial improvement will not happen by self-determination alone. If we seriously want to increase the number of foreigners and females as tenured faculty members of our universities, we should consider affirmative action programmes. If we really want young scientists to be more active, we should even consider introducing medium-term contracts to give them more opportunity and responsibility.

Implementing such proposals may require amendments to current laws. However, whatever commitments are made by legislators, the unacceptable academic discrimination that now exists in Japan will not disappear until faculty and administrators in the universities themselves genuinely recognize the need for reform. □

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Unacceptable academic discrimination will not disappear until faculty and administrators in the universities recognize the need for reform

A molecular handle on the Neanderthals

Ryk Ward and Chris Stringer

The sequencing of mitochondrial DNA from Neanderthal fossil bone is a terrific achievement. Among the conclusions to be drawn from the data is that there was a period of more than 500,000 years during which Neanderthals and the line leading to modern humans evolved independently.

Neanderthals hold a central place in studies of human evolution, yet their relationship to contemporary humans remains enigmatic^{1,2}. Now, a molecular analysis has seemingly resolved many of the disputes about that relationship. The work, reported in last week's *Cell*³, represents the first successful recovery and analysis of mitochondrial DNA (mtDNA) from a Neanderthal specimen. Even more remarkably, the sequence was recovered from the first Neanderthal skeleton described, the type specimen, one of the most important human fossils ever found.

The type-specimen Neanderthal skeleton was discovered in the Neander Valley, near Dusseldorf in Germany, and described in 1857 (see refs 1 and 2 for an overview). This event ignited an intense debate about the degree of morphological distinctiveness — and biological continuity — between ancient and modern humans. Some early workers believed that the peculiar aspects of Neanderthal morphology were due to skeletal diseases. But additional finds made it clear that such remains represented a population of archaic humans which inhabited Europe and western Asia between about 230,000 and 30,000 years ago.

In agreement with what we know of their environments and lifestyle, their physique was muscular and thickset, with body proportions apparently reflecting adaptation to cold conditions and with a skeleton built for strength and endurance. Although the morphological diversity of the Neanderthal population was probably at least as great as that of modern humans, overall they were characterized by a number of distinctive anatomical features^{1,2}.

More recently, even older types of archaic humans have been found that are morphologically distinct from both Neanderthals and contemporary human populations. Their presence in Europe and Africa only serves to increase the complexity of untangling the threads of human evolution. As far as Neanderthals are concerned, it is difficult to find consistent morphological antecedents in these early remains. Although some workers claim that Neanderthal morphology runs back to the early Middle Pleistocene, some 600,000–800,000

years ago, others argue for their genetic continuity with modern humans. An intermediate position is that a Mid-Pleistocene species (*Homo heidelbergensis*), which occurred in Africa and Europe, gave rise to both Neanderthals and modern humans⁴.

The new molecular work³ considerably clarifies matters. But it is first necessary to realize that the difficulty of working with ancient DNA (see box, overleaf) required many procedures to generate the primary data. The first step evaluated the amount of amino-acid racemization, as a surrogate measure of the amount of damage likely to have been suffered by the Neanderthal DNA. Although the degree of racemization of aspartic acid was approximately double that of modern bone (indicating some degradation), the results lay within the range of feasible amplification with the polymerase chain reaction. Two independent PCR reactions were accordingly performed, using primers designed to amplify a short, 105-base-pair segment of the hypervariable segment of the human mtDNA control region. In each case, a PCR product was obtained and these were cloned into plasmid vectors and 30 clones sequenced.

The resulting sequences indicated that the PCR products contained a mixture of molecules (see box): a small minority population of modern human sequences, presumably contaminants, and a greater number of highly distinctive sequences, tentatively identified as the Neanderthal sequence. The putative Neanderthal sequences were characterized by the presence of eight deviations from the reference sequence (out of 61 positions). As the same set of eight substitutions was obtained on two further PCR amplifications, it was highly likely that this sequence came from the Neanderthal fossil.

Because primers specifically designed to amplify the putative Neanderthal sequence (rather than modern human mtDNA) failed to yield products from a sample of modern humans, the possibility that the variant sequence might be a nuclear insert could be eliminated. Finally, and perhaps most importantly, the putative Neanderthal sequence was independently replicated in a collaborating laboratory, so this mtDNA

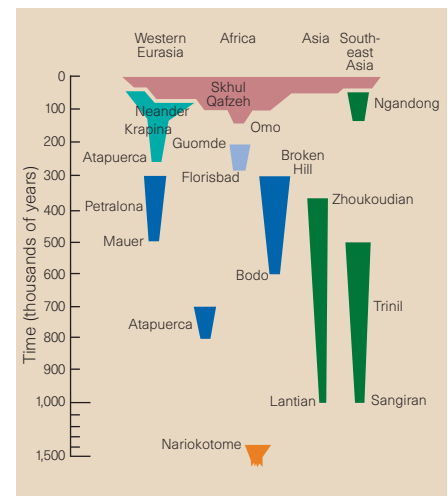


Figure 1 Distribution in time and space of the various human lineages that evolved from *Homo ergaster* (orange), the first expansion leading to the establishment and subsequent development of *H. erectus* lineages (green) in Asia and Southeast Asia. Other lineages include three groups of *H. heidelbergensis* (dark blue), Neanderthals (turquoise), a transitional group of archaic *H. sapiens* in Africa (light blue), and modern *H. sapiens* (mauve), the latter ultimately eclipsing all other human lineages. The new Neanderthal mtDNA results³ support the hypothesis that the original divergence that eventually led to Neanderthals and modern humans began more than 500,000 years ago in *H. heidelbergensis*. Places mentioned are sites of significant fossil discoveries.

sequence is almost certainly derived from the Neanderthal specimen itself.

To characterize a longer sequence, one likely to be more informative in evolutionary terms, a series of 13 independent overlapping amplifications (see box) was carried out, and the products cloned. After aligning the resulting set of 123 sequences from individual clones, a consensus sequence of 379 base pairs was obtained. This sequence exhibited 27 differences from the reference sequence, compared to the 5–8 differences expected from a random sample of modern humans. Detailed comparisons of the Neanderthal sequence with 994 human mtDNA lineages and 16 chimpanzee mtDNA lineages indicated that, although human mtDNA lineages differed among themselves by 8.0 ± 3.1 substitutions, the difference between humans and the Neanderthal sequence was 27 ± 2.2 . The overall difference between the Neanderthal sequence and that of humans was about three times the average difference among humans, but only about half the difference between humans and chimpanzees.

This implies that the Neanderthal divergence is of considerable antiquity, dating to 555,000 to 690,000 years ago. This is about four times greater than the time back to the common ancestor of modern human mtDNA (120,000 to 150,000 years). ▶

Standards for research on ancient DNA

It is the development of sound experimental procedures, rather than the delivery of breathtaking but dubious results, that has represented the key to establishing the credibility of research on ancient DNA. Aside from taking due care with the extraction procedure, and controlling for contamination, four essential experimental procedures should be carried out.

- Determine the amount of change that has occurred and resulted in damaged or degraded DNA. Because the rate at which L-amino acids undergo racemization to D-amino acids is dependent on much the same factors that induce DNA damage (water, temperature), the D/L ratio of amino acids can be used as an indirect measure of the extent of DNA damage⁷. Typically, a D/L ratio greater than 0.08 suggests that DNA cannot be reliably retrieved from the sample.

- Determine the number of intact template molecules in the polymerase chain reaction. Too few template

Position:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Reference sequence:	A	C	T	A	T	T	A	A	T	C	C	T	A	C	G
Clone															
A.1	.	.	C	G	.	.	G	.	.	.	.	.	.	.	.
A.2	G	.	C	G	.	.	G	.	.	.	.	.	.	.	.
A.3	.	.	C	G	.	.	G	.	.	.	.	.	.	.	.
A.4	.	.	C	G	.	G	.	.	.	.	.	.	.	.	.
A.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B.1	.	.	.	.	C	.	G	.	C	.	T	.	.	.	.
B.2	.	.	.	.	.	G	.	C	T	T	.	.	.	.	.
B.3	.	.	.	.	.	G	.	C	T	.	.	.	.	.	.
B.4	.	.	.	.	.	G	.	C	T	.	.	.	.	.	.
B.5	.	.	.	.	.	.	G	C	.	.	.	.	.	.	.
C.1	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.
C.2	.	.	.	.	.	.	.	.	.	T	.	.	T	A	.
C.3	.	.	.	.	.	.	.	.	.	T	.	T	T	.	.
C.4	.	.	.	.	.	.	.	.	.	T	.	.	T	.	.
C.5	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Ancient DNA sequence	A	C	C	G	T	T	G	A	C	C	T	T	A	T	G

molecules will yield unreliable and inconsistent results, because of the misincorporation of nucleotides and other artefacts of PCR. However, once the correct target has been identified, a diagnostic template can be synthesized and serial dilutions set up for quantitative PCR experiments, yielding estimates of the number of original template molecules⁸. PCR reactions with substantially fewer than

50–100 template molecules tend to be highly suspect.

- Use cloning to identify the composition of the PCR products, and to obtain a consensus sequence for the target. Cloning the PCR product³ reveals its underlying nature and allows reconstruction of the ancient DNA sequence. The figure here illustrates five clones from each of three independent, overlapping, amplifications (A, B, C). In each amplification there is a

minority sequence (A.5, B.5, C.5) resembling the reference, and a presumed contaminant. Alignment of the remaining 12 sequences allows reconstruction of the ancient DNA sequence and indicates that it deviates from the reference at six positions (3, 4, 7, 9, 11, 14). Note that the modern contaminant has a polymorphism at position 9, and that there are six instances of nucleotide misincorporation (singleton bases in green).

- Use independent replication to confirm results. This is a standard experimental procedure, but all too often it has been ignored in work with ancient DNA.

The collective application of these four criteria not only marks the coming-of-age of research on ancient DNA, but serves as a useful yardstick for investigator and reviewer alike. In this light, the new results on Neanderthal mtDNA are merely a tantalizing indication of the landmark contributions that can be expected over the next decade. **R.W. & C.S.**

More extensive analyses confirmed that the Neanderthal sequence consistently fell outside the mtDNA sequence variation observed in modern humans. They also suggested that the closest contemporary lineages to the Neanderthal sequence came from Africa. So the genetic relationship between Neanderthals and modern Europeans appears to be no closer than the average relationship between Neanderthals and any modern human — running counter to the view that Neanderthals were at least partly ancestral to modern Europeans⁵.

These molecular results, then, imply that there was a period of roughly half a million years of independent evolution of Neanderthals and the line leading to modern humans. This, in turn, suggests that the divergence might have occurred at about the time that the two main lines of *Homo heidelbergensis* became distinct in Europe and Africa respectively (Fig. 1). Such a scheme of events implies that the Mid-Pleistocene forms of *H. heidelbergensis* in Europe may have eventually led to early Neanderthals, such as the specimens from Atapuerca in Spain, and ultimately to

the 'classic' forms found at Krapina, in Croatia, and at Neander itself. Conversely, modern humans are likely to have evolved from transitional groups of archaic *Homo sapiens*, such as those found at Guomde and Florisbad, in East and South Africa respectively, which in turn were independently derived from later *H. heidelbergensis* forms in Africa. Intriguingly, still other forms of humans may have survived in Southeast Asia⁶ (see Fig. 1).

With respect to the contentious issue of whether Neanderthals and anatomically early modern humans exchanged genes, these new results diminish, but do not rule out, that possibility. Clearly, Neanderthal populations represented by the type specimen did not contribute mtDNA to the modern human population. But, as the authors are careful to point out, this does not exclude the possibility of exchange of nuclear genes. In our view, whether we choose to designate them as separate species or not, half a million years of independent evolution of two highly specialized forms of human, with all the attendant behavioural implications, make such an exchange unlikely. Unfortunately, given the

extremely low probability of obtaining nuclear DNA from the Neanderthal specimen, this is a hypothesis that may never be directly tested.

Given the quality of the new molecular findings, the study of ancient human DNA can, at long last, be said to be on a secure footing. As additional molecular data of increasing complexity are produced, the near future looks set to be a truly revolutionary time for students of human evolution. □

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Volcanology

The giant Popocatépetl stirs

Servando De la Cruz-Reyna and Claus Siebe

On 30 June, Popocatépetl showered ash over Mexico City, about 72 km away, in its largest eruption since 1927. This comes after a few years of increasing activity, which could conceivably be the precursor of a huge eruption.

Popocatépetl, a 5,452-metre-high volcano, is in one of the most densely populated areas in the world. For centuries, its low eruption rate, the fertile volcanic soil, and the mild climate of the high plateau have favoured population growth on hazardous areas around the volcano. Currently, over one hundred thousand people dwell in an area that could be directly affected in the event of a major eruption, and nearly 20 million live within a radius of 80 km, where they are threatened by ash falls.

In 1993, after nearly 70 years of quiescence, Popocatépetl volcano reawakened, with increased seismic activity and gas emission from fumaroles.

This activity increased in late 1994, culminating in the early hours of 21 December in a series of small explosions at the crater. These explosions were followed by emissions of ash, which fell on several towns to the northeast of the volcano and on the city of Puebla. Around 75,000 people were evacuated from the most vulnerable towns. Pulsating ash emissions, lasting 5–20 minutes and occurring a few times a day, persisted through 1995. In March 1996, a lava dome was observed growing on the floor of the crater, and currently this lava has filled about 20 per cent of the crater's volume.

Although the lava extrusion rate seems to have decreased, ash emissions continue. Some emissions are more explosive. In April 1996, a small explosion killed five climbers trying to photograph activity at the crater rim, ignoring warnings. During 1996 and 1997, small explosions have thrown incandescent blocks of lava onto the upper slopes of the volcano. And over the past three years, Popocatépetl has been one of the largest SO₂ producers in the world. It has probably been acting as an open or semi-open system, thus allowing some sort of equilibrium to exist between the energy fed from deeper sources and that dissipated by exhalations and similar, medium- to low-level activity.

Today, we know that the present cone is not the first high volcanic edifice at this site. Geological evidence points towards a tremendous eruption 23,000 years ago, which destroyed the previous volcano. Since then, there have been many small eruptions and at least seven catastrophic eruptions which each produced several km³ of ash and pumice. The three most recent of these occurred within the period of human occupation, at about 3000 BC, 200 BC and AD 800, as shown by archaeological remains buried by ash, and pottery shards incorporated by mud flows. (The famous archaeological sites of Cacaxtla and Cholula were severely affected in this way by the AD 800 eruption.) Since AD 800, activity at Popocatépetl has been moderate.

The present episode has many similarities to the 1919–27 period of activity, which ended with the explosive destruction of the dome, forming a small crater on the floor of the main crater. The present dome has already exceeded the volume of the 1919–27 dome, although it may take several years to grow large enough to reach the lowest part of the crater rim. It has grown in a pulsating manner.

Because the area affected by prehispanic eruptions is today densely populated, Popocatépetl deserves the utmost respect and attention. Whether the current evolution will end without exceeding the levels of explosivity of the past few hundred years, or whether it leads to an eruption as large as the catastrophic event 1,100 years ago, is a question we cannot answer yet. Probability favours the former eventuality, but a larger eruption cannot be ruled out.

For this reason, an elaborate surveillance system has been established, which includes real-time seismic monitoring and observations of ground movements and chemistry; and a 'traffic light' alert code has been devised, which defines the responsibilities of the involved parties, namely, a scientific committee, the civil protection authorities and the population in danger. This code includes a simplified decision-making scheme, in which the number of options that may be taken in a particular volcanic crisis is reduced to a minimum. These colour-coded options are chosen, for each one of the hazardous regions defined by the volcanic risk map, according to the level of activity as judged by the scientific committee.

The decision-making involved in these colour-coded conditions is proving to be a difficult task. At present, the alert is at 'yellow'. The challenge is to recognize the earliest precursors that would necessitate changing the alert to a condition that may involve the evacuation of tens of thousands of people. Generally speaking, such a process involves comparison with other volcanoes. However, Popocatépetl has shown that volcanoes cannot be compared indiscriminately. Indeed, some of the observed parameters — namely, magmatic SO₂ gas production — have exceeded by a large factor levels observed at other volcanoes during their pre-eruptive phase, and even during intense explosive activity. So the main question remains unanswered. Is the present activity only another small intermezzo? Or are we witnessing the precursors of a large explosive eruption? □

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Figure 1 Ash emission earlier this year at Popocatépetl. In the background is Iztaccíhuatl, an older volcano to the north.

Spongiform encephalopathies

Tracking turncoat prion proteins

Colin L. Masters and Konrad Beyreuther

The persistent fear that bovine spongiform encephalopathy (BSE, or 'mad cow' disease) has crossed species barriers into humans and other animals has increased the pressure on scientists to come up with a molecular explanation for infectivity. Two *in vitro* experiments, one assaying the conversion of purified mammalian prion protein (PrP) to its abnormal form in a cell-free system (reported by Raymond *et al.* on page 285 of this issue¹) and the other investigating the polymerization of a yeast prion-like protein (described in *Cell* by Glover *et al.*²), now provide further evidence for the 'protein-only' hypothesis, in which transmission depends on the inheritance of an altered protein rather than nucleic-acid structure. The PrP studies also confirm in molecular terms that the empirically observed species barriers do indeed exist, although the strength of these barriers in determining the spread of disease remains uncertain.

For the mammalian transmissible spongiform encephalopathies (BSE in cattle; scrapie in sheep; Creutzfeldt–Jakob disease (CJD) and kuru in humans), the

host-encoded PrP glycoprotein is transformed into a relatively insoluble and protease-resistant (PrP-res or PrP^{Sc/CJD/BSE}) conformer of the normal protease-sensitive PrP (PrP-sen, or PrP^C). This transformation involves a shift in conformation from α -helix to β -sheet (Fig. 1), and the operational detection of PrP-res is the principal method currently used for studying this process *in vitro*.

Two hypotheses have evolved to account for this process: template direction and nucleated crystallization (Fig. 1). It is likely that at least one other factor (protein X) is involved in the generation of metastable oligomeric complexes of PrP, which may, under certain conditions, polymerize into amyloid fibrils. Infectivity and the manifestation of disease (neurotoxic vacuolation and degeneration) have an absolute requirement for the presence of PrP^C (for reviews see refs 3, 4).

The phenomena of strain variation and species specificity are presumed to depend on the efficiency and reproducibility of the conversion of PrP^C to PrP-res. In an earlier cell-free conversion assay⁵, a difference in

the efficiency of conversion between mouse and hamster was detected, which partly reflected the interspecies barrier. Raymond *et al.*¹ have now demonstrated a clear decrease in the conversion efficiency of heterologous PrP reactions (cow $\rightarrow$ human; sheep $\rightarrow$ human) compared with intra-species homologous (mouse $\rightarrow$ mouse, for example) PrP reactions for mouse, hamster, sheep, cow and human.

Do these findings tell us anything about the molecular basis of these interactions? Preliminary data show that the conversion efficiency of PrP^C does not depend on its glycosylation, which is consistent with the endoplasmic reticulum being the site of the earliest change in the conversion to PrP-res (ref. 6). The assay also indicates that certain amino-acid residues (for example, the one encoded by codon 129 in humans) are crucial for the conversion, being the same residues that determine the genetic basis of infectivity. However, the cell-free assay does not distinguish a true PrP^C $\rightarrow$ PrP-res conversion (that is, the *de novo* creation of infectivity) from a protective binding event in which PrP^C achieves relative protease resistance by its close binding to PrP-res. Cell-associated assays have been designed to detect PrP-res (ref. 7), however, and these address many of the weaknesses of the cell-free assay. Between them, both assay systems should enable the structural determinants that are involved in the conversion reaction to be thoroughly analysed.

Can the general public take any comfort from this new evidence that the efficiency of the BSE-driven conversion of human PrP to a pathological form is comparable to that of sheep scrapie? After all, Europeans have been living in close association with sheep scrapie for centuries, with no evidence of its having been transmitted to humans. Although it is reassuring to know that the transmissibility characteristics of BSE and sheep scrapie appear to be similar *in vitro*, other factors such as dose and route of inoculation and the effect of protein X may influence the potential of BSE to spread across species barriers.

The *in vitro* conversion assay for mammalian PrP has not yet been able to shed much light on the underlying molecular processes, but the study by Glover and colleagues² of the yeast [PSI⁺] factor strongly supports a mechanism for conversion that is based on nucleated crystallization (Fig. 1). The cytoplasmically inherited factors [PSI⁺] and [URE3] of yeast share many of the features of the mammalian prion system^{8,9}. Sup35 is the protein determinant of the [PSI⁺] phenotype. In [PSI⁺] strains, Sup35 is found to aggregate and become insoluble. Purification of Sup35 shows that it is highly amyloidogenic, forming β -sheet fibrils with at least two distinctive (smooth and wavy) self-perpetuating morphol-

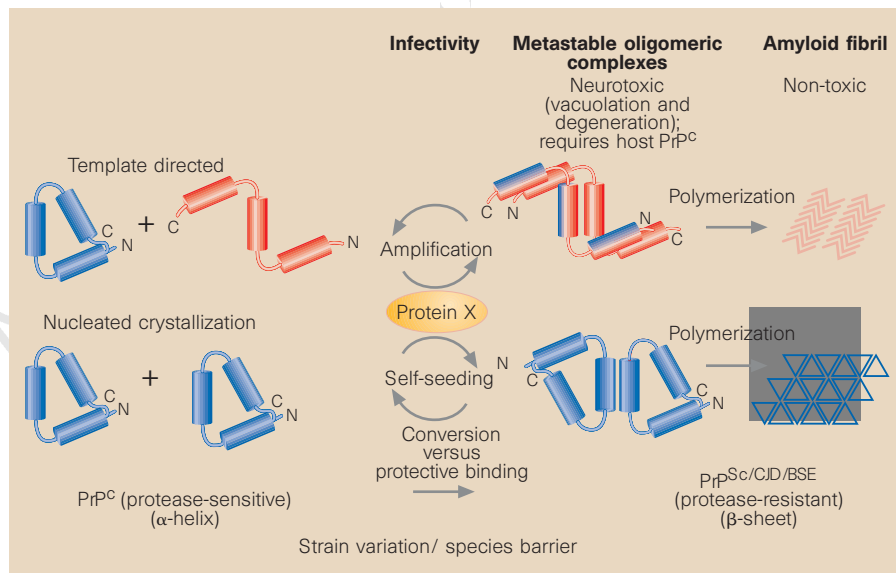


Figure 1 Two hypotheses attempt to explain the phenomenon of a self-replicating infectious protein. Top, the template-directed model, in which the normal isoform of PrP (shown in blue as a triangular-shaped backbone resembling the α -helical domains determined by nuclear magnetic resonance¹²) encounters an abnormal conformer of PrP (pink). This engenders a metastable oligomeric complex in which the normal isomer is induced to assume a similar conformation. Either the forward conversion reaction is modulated by protein X, or the abnormal conformer is maintained by protein X. Disease results from the toxic effect of the metastable complex. Further polymerization of the complex may result in amyloid fibril formation. Bottom, the nucleated-crystallization model, in which the self-seeded oligomeric complex is dependent on the self-perpetuating properties of the oligomeric complex, which may also result in amyloid fibril formation. Both hypotheses require the operational conversion of PrP^C (the protease-sensitive, predominantly α -helical form) to PrP^{Sc} (the protease-resistant, predominantly β -sheet form). In both models, there is an absolute requirement for PrP^C to maintain the cycle of conversion and hence infectivity.

ogies². The kinetics of fibril formation and the effect of seeding in both purified and *in vivo* preparations support the nucleation self-seeding hypothesis.

The [PSI⁺] phenotype is also dependent on the expression of a heat-shock protein (Hsp104), a chaperone that oversees protein folding, which may therefore subsume the role of protein X. It is possible that if the concentration of such a heat-shock protein were drastically increased as a result of local stress, a nucleating seed could be created. If different heat-shock proteins were to form different 'strains' of seeds, then the basis of strain variation could be found in the structural organization of this nucleating molecule. Once the formation of Sup35 fibrils is initiated, a constant morphology is maintained, so it will be interesting to see whether Hsp104 plays a part in this effect.

Further support for the nucleation hypothesis has recently come from a mammalian system in which compounds that bind nonspecifically to amyloid fibrils affect the progression of experimental scrapie¹⁰. But the discussion is by no means over. The strongest evidence for a template-directed process is found in the persistence of strain differences in the physical characteristics of PrP-res upon transmission¹¹. In the final analysis, it may be that the models are not mutually exclusive, or that both mechanisms are involved.

Has BSE crossed the species barrier into humans, and are the 'new-variant' CJD cases, few though they are, a harbinger of future trends? The new molecular *in vitro* data^{1,2} should help us understand how on the one hand we are dealing with diseases that generate high titres of infectivity, but on the other are not particularly contagious. If we're lucky, the species barrier, encoded at least in part within the amino-acid sequence of PrP, will prove to be self-limiting. But if we're unlucky ...

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Symbiosis

Making light work of adaptation

Robert W. Buddemeier

Reef-building corals are photogenic 'doe-eyed invertebrates' which form high-diversity, shallow-water communities that are often loosely analogized to rainforests. They are cnidarians — relatives of jellyfish and anemones — and are usually, but not invariably, colonial. Among their distinguishing characteristics are the algal (dinoflagellate) symbionts that live within their tissues, and the rapidity with which their calcium carbonate (aragonite) exoskeletons are formed.

Because reef-building corals are vulnerable to human-induced stress, resulting in the mysterious phenomenon of 'bleaching' (stress-induced loss of the pigmented algae, with sometimes fatal consequences)¹, international monitoring and assessment groups have been set up^{2,3}. The paper by Rowan *et al.*⁴ on page 265 of this issue now opens up new pathways to understanding this complex biological system. The authors convincingly extend previous work, showing that there is neither a single taxon of symbiotic algae, nor a uniquely obligate relationship between a host species and a single algal taxon. Rather, at least some symbiotic corals are communities, and the distribution of the various plant taxa is controlled by the levels of light that are available in the microenvironment. Moreover, a synergistic relationship between light and temperature controls the distributions of algal taxa within the coral colony. Under high-temperature stress, the algal symbionts at the upper end of their normal irradiance range are preferentially lost (bleached). And the new work implies that the relationship is somewhat symmetrical — at marginally stressful temperatures, we would expect increases in irradiance to have similar effects.

Photosynthesis by the symbiotic algae consumes carbon dioxide. Marine calcification precipitates carbonate ions, forcing a re-equilibration of the bicarbonate-dominated marine inorganic carbon system that creates a carbon dioxide source⁵. So, it has long been an article of geological faith that the coral symbiosis is intimately and causally linked to the high rates of calcification in reef-building corals, compared with the rates in asym-biotic, non-reef-building corals⁶. This is also thought to have led to the massive accumulations of carbonates in shallow-water environments, and possibly even to feedback-controls on the global carbon cycle⁷.

Coral reefs are restricted to shallow-water tropical and subtropical environments, which are characterized by warm temperatures, aragonite supersaturation (concentra-tions of calcium and carbonate ions that

exceed the thermodynamic mineral solubility product), and high light intensities⁸. Along with this biogeography, the fidelity with which corals' aragonite exoskeletons record the isotopic and chemical characteristics of their growth environment⁹ has made them favourites of the marine palaeoclimate research community. Because corals have been around for more than 200 million years, and seem to be relatively stable in terms of rates of speciation and extinction¹⁰, the many recognizable fossils have made a large contribution to our understanding of climates and oceans past.

The current paradox of coral-reef research is that the organisms and ecosystems involved seem to be vulnerable to environmental change in the short term, yet they have persisted over hundreds of millions of years, through rapid (and sometimes extreme) environmental change — so how do they do it?

The demonstration by Rowan *et al.*⁴, of labile and micro-adaptive symbiotic combinations, fits well with other recent revisions of the conventional wisdom regarding coral systematics. Veron¹⁰ has reported that corals undergo reticulate evolution, characterized by hybridization and a level of genetic variation that is more common in plants than in

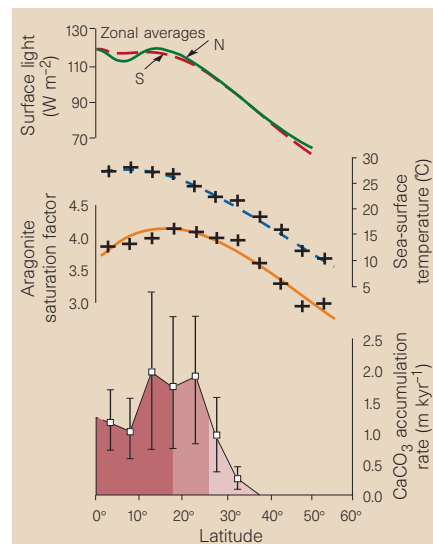


Figure 1 Latitudinal distributions of Holocene calcium-carbonate accumulation rates and types (shading indicates the relative importance of coral-algal communities), compared with temperature, surface-water aragonite saturation ratio⁷ and photosynthetically active surface radiation (400–700 nm)⁸. The existence of a 'tunable' multi-component algal symbiosis, proposed by Rowan *et al.*⁴, suggests that all three environmental variables play interacting roles in coral physiology and biogeography.

higher animals. Fautin¹¹ points out that varied reproductive strategies and the heritability of somatic mutations may result in considerable genetic variability, even within the presumed single clone of a large colony. So, many (if not all) coral 'species' represent a palaeontologically stable 'morphospecies', with genetic, symbiotic and niche variabilities that make a mockery of attempts to apply the species concepts that are associated with higher animals and terrestrial organisms.

The significance of these findings goes well beyond the level of depressing (but enlightening) news for organismic coral researchers — whether their bailiwick is the past or the present. The mechanisms by which corals calcify are not well understood, but it has long been known, for example, that skeletal morphology changes in many species as a function of the levels of light. Moreover, the isotopic and chemical composition of the exoskeleton may vary with the rate of growth (extension). Similar variations in how the taxa of the algae that mediate calcification are distributed within a colony constitutes a map for future research — not just an interesting, but frustrating, anomaly. Even more fundamental to the relationship between light, temperature and calcification are studies that seem to uncouple coral calcification¹² or extension¹³ from symbiosis or light, despite earlier experiments which showed that light/dark calcification ratios in symbiotic corals are much greater than unity¹⁴.

The discovery of many labile symbioses by Rowan *et al.* may help to resolve the apparent paradox: if reef-building corals retain the same, genetically hard-wired calcification mechanism that allows their asymbiotic cousins to build skeletons in dark, cold, undersaturated water, this may be reflected in the 'dark calcification' that builds the skeletal template¹³. In the light, photosynthesis by a 'tunable' suite of algal symbionts helps to optimize the harvest of calcium carbonate from supersaturated waters. This calcification component may even be as important as temperature in explaining why reefs are mainly in the tropics (Fig. 1). And the concept of dual-process calcification is a diel analogue of the differential extension-thickening model that explains variations of skeletal density on a seasonal basis¹⁵.

The present spate of exciting and unsettling findings highlights the roles of acclimatization and adaptation in explaining how the consistent metabolism of coral communities over space and structure¹⁶, and the consistent structure of these communities over time¹⁷, can be achieved by evolutionarily stable taxa in a highly variable environment. And these seemingly arcane questions are central to more immediate and practical issues, such as natural controls on carbon cycles and the survival of ecosystems and

biodiversity during global change. Whether they like it or not, coral researchers are living in interesting times. □

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Immunodeficiency viruses

Spoilt for choice of co-receptors

Paul R. Clapham and Robin A. Weiss

As intracellular parasites, viruses need first to attach to and then gain entry into cells. The human and simian immunodeficiency viruses (HIV, SIV) carry out these steps through surface glycoproteins which interact with different molecules displayed by the host cell. All immunodeficiency viruses bind with high affinity to the CD4 cell-surface receptor of T lymphocytes, which attaches them to the cell under attack, but different strains use distinct co-receptors to trigger viral entry (see Fig. 1 and our previous News and Views article¹ on the topic).

In papers on pages 296 and 238 of this issue^{2,3}, and elsewhere^{4,5}, several groups announce the discovery of further co-receptors for SIV and HIV infection; indeed, a receptor molecule encoded by cytomegalovirus is also reported to serve as an HIV co-receptor⁶, although this may not hold for all cytomegalovirus strains. The ability of immunodeficiency viruses to use so many receptors has provoked much thought about HIV pathogenesis and how it might be prevented.

CD4 was identified in 1984 as the receptor for HIV, but it soon became apparent that its presence alone was not sufficient to permit viral entry. The identity of second receptors remained elusive until 18 months ago, but since then it has become clear that there is a growing family of them, used by different HIV and SIV strains. Their expression on different (but often overlapping) cell populations, along with selective use by particular virus strains, makes them a major determinant of which cells HIV can infect.

All the co-receptors so far identified are members of, or related to, the seven-transmembrane, chemokine-receptor family. These are G-protein-coupled signalling receptors which bind the chemoattractant proteins, known as chemokines, that are involved in controlling activation and

migration of various leukocytes. All HIV-1 strains so far tested use the CCR5 or CXCR4 co-receptors, or both, and therefore these two are likely to be crucial to HIV infection and pathogenesis *in vivo*. Many HIV-1 strains, however, are able to use other co-receptors — including CCR3, CCR2b (refs 7, 8) and three of the four newly identified ones (see Table 1) — as well as CCR5 or CXCR4.

The CCR5 co-receptor is targeted by the so-called non-syncytium-inducing (NSI) viruses of HIV-1, those that don't induce cell-to-cell fusion in T-cell lines. It seems crucial for viral transmission because people who are homozygous for a CCR5 gene deletion are largely protected from HIV-1 infection⁹. CCR5-using strains are usually present throughout the course of infection. Syncytium-inducing (SI) viruses, by contrast, use the CXCR4 co-receptor, and often emerge later in the course of infection; they can be isolated from about half of AIDS patients.

In the co-receptor context, SIV presented a puzzle because some strains infect several CD4-positive human and non-human cell types that are resistant to HIV-1 (ref. 10). That puzzle grew more complex earlier this year when it was found that SIV used CCR5 but not CXCR4 (refs 11–13), yet could infect the CCR5-negative human T-cell lines Hut 78 and CEM × 174, as well as the astroglial cell line U87.CD4. Clearly, these strains used different and unidentified co-receptors.

Deng *et al.*² now describe two new co-receptors for SIV. They have been identified through screening complementary DNA libraries for their capacity to allow SIV entry into cells, and are termed Bonzo and BOB (standing for 'brother of Bonzo'; alas, the names will change to the more conventional terminology when their natural ligands are identified). Both receptors were used by the

Table 1 Immunodeficiency virus receptors

	CD4	CCR5	CXCR4	CCR3	CCR2b	Bonzo STRL33	BOB GPR15	GPR1	US28
HIV-1 NSI	+	+	–	+	–	+	+	–	+
SI	+	+	+	+	+	(+)	+	–	+
SIV _{mac}	+	+	–	–	–	+	+	+	?
Natural ligands	MHC class II	MIP-1 α MIP-1 β RANTES	SDF-1	Eotaxin RANTES MCP-3 MCP-4	MCP-1 MCP-2 MCP-3	?	?	?	?

All strains of HIV and SIV bind to CD4. Eight co-receptors are shown that bind different HIV and SIV strains. +, indicates that the appropriate co-receptor is active for at least one virus strain. –, means that no viruses have yet been shown to use that co-receptor. ?, means not yet tested or unknown. The known natural ligands of co-receptors are all chemokines. NSI, non-syncytium-inducing, SI, syncytium-inducing. The newly identified co-receptors are Bonzo/STRL33 (refs 2–4), BOB/GPR15 (refs 2, 5), GPR1 (ref. 5) and US28 (ref. 6).

three strains of SIV tested, and by several HIV-1 and HIV-2 strains. BOB is identical to a receptor (GPR15) described in 1996 (ref. 14), but not hitherto linked to HIV/SIV infection. Bonzo is identical to a co-receptor (STRL33), described last month by Liao *et al.*⁴, which functioned as a co-receptor for all of eight HIV-1 strains tested (both NSI and SI). The same group³ now add that the Rhesus macaque SIV (SIV_{mac}) uses STRL33/Bonzo. In addition, Farzan *et al.*⁵ show that two orphan molecules^{14,15}, resembling chemokine receptors, are used by SIV_{mac}. GPR15 (from CEM $\times$ 174 cells) is the same as BOB, whereas GPR1 (from U87.CD4 cells) is novel as a virus receptor.

In all, then, three new human co-receptors for immunodeficiency viruses have been identified — BOB/GPR15, Bonzo/STRL33 and GPR1. Each can be used by the SIV_{mac} strains tested, but although GPR1 is, so far, specific for SIVs, BOB and Bonzo can be used by several HIV-1 strains. Farzan *et al.*⁵ and Deng *et al.*² agree that only certain HIV-1 strains target BOB. Deng *et al.* report a more restricted use of Bonzo than do Liao *et al.*⁴, with entry by some but not all primary HIV-1 isolates. Despite this minor discrepancy, Bonzo and BOB could be important in both HIV and SIV infection *in vivo*. BOB and Bonzo are expressed widely in lymphoid tissue, the main site for HIV and SIV replication². Bonzo but not BOB messenger RNA was present in monocytes, whereas GPR1 was detected in alveolar

macrophages but not T cells⁵. High levels of BOB mRNA were detected in colon tissue, prompting Deng *et al.* to speculate on a possible role in HIV or SIV transmission to or from this site.

What are the implications of these new results? First, the lack of CXCR4 use by SIV strains, along with the possibility that BOB, Bonzo and particularly GPR1 might be more significant for infection by SIV than by HIV-1, mean that SIV may target distinct haematopoietic cell populations; if so, some caution is called for in extrapolating from SIV infection of macaques to HIV-1 infection of humans. But it is still unclear how many HIV-1 strains infect cells through BOB and Bonzo, and whether GPR1 is

used at all. Moreover, many HIV-positive patients, like SIV-infected macaques, develop AIDS without the appearance of CXCR4-using SI strains.

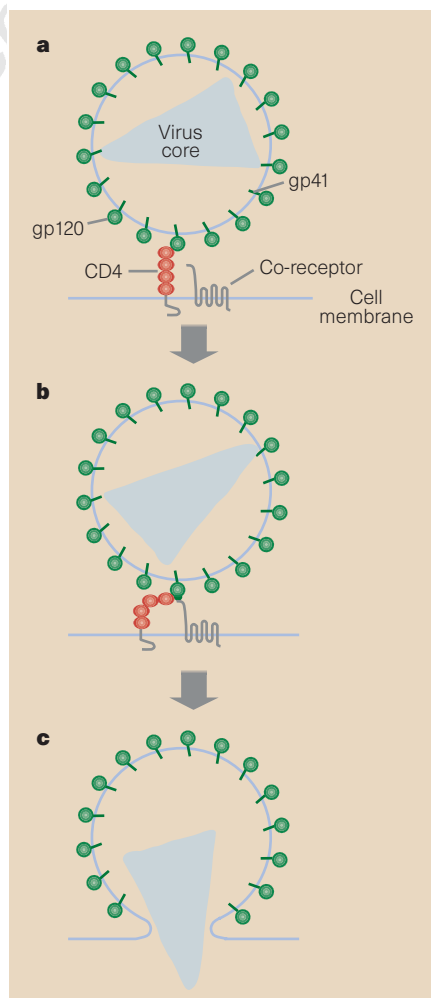
Second, there are the implications for drug development. The discovery of co-receptors has brought hope that new drugs can be developed that block HIV-1 entry into cells. Modified chemokines can indeed inhibit HIV infection by competing with HIV for specific co-receptors, apparently without activating the immune system^{16,17}. But the growing number of HIV-1 co-receptors being identified implies that targeting specific co-receptors may simply mean that other HIV strains using alternative co-receptors emerge and come to predominate. It should not be forgotten, though, that whereas we can assess the extent of co-receptor use in cell culture, we cannot yet do so *in vivo*; and that we don't fully understand the selective *in vivo* pressures (innate or immune) that act for or against the use of individual co-receptors.

Six human co-receptors that are active for HIV-1 strains are now known (see Table 1). More will probably follow. The capacity of evolving HIV/SIV strains to use additional co-receptors is likely to provide them with new cell populations to target, as seen for SI viruses that use the CXCR4 co-receptor and tend to emerge late in disease. CXCR4 expression is more widespread, and includes naive T cells, while CCR5 is mainly expressed on macrophages and memory T cells¹⁸. It remains controversial whether this change in co-receptor use (to CXCR4), and therefore type of T cell infected, results in the precipitous drop in CD4-positive T cells that often follows the emergence of SI HIV-1. Much careful work is now required to establish the contribution of each co-receptor to HIV infection and pathology.

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Figure 1 HIV and SIV attachment and entry into cells. Spike glycoproteins (gp) on virus particles are made up of surface gp120 noncovalently attached to transmembrane gp41. Each spike contains three of each. a, gp120 binds to CD4 at the cell surface to attach virus particles to the cell. b, Conformational changes in the spike glycoproteins generated by their interaction with CD4 result in co-receptor binding. Further rearrangements in the spike glycoprotein induce the exposure of a hydrophobic domain at the amino terminus of gp41, and insertion of this region into the cell membrane initiates fusion of the cell and viral membranes (see ref. 19). c, After fusion, the virus core containing the RNA and reverse transcriptase enters the cytoplasm of the cell.





100 YEARS AGO

In our issue for June 24 we briefly described the run of the *Turbinia* from the Tyne to the Solent. We understand that during the three weeks the *Turbinia* was in the Solent she made frequent runs of many miles at a time, at speeds of from 30 to 35 knots On Tuesday, June 29, with a distinguished company on board, she was run up to nearly full power, and maintained the unprecedented speed of 35 knots, or over 40 miles per hour, for the length of the line of battle-ships, or about 5 miles. During this run there was an absence of strain, and from this fact it seems that the limit of speed in this little vessel has not yet been reached, and that after further improvements, at present in progress (having returned to the Tyne last week), she will be capable of not only maintaining her position as much the fastest vessel afloat, but will be able to give many knots to any competitor engined with reciprocating engines.

From *Nature* 15 July 1897.

50 YEARS AGO

A description of developments [in television] was given in a lecture on May 29 before the Television Society by Dr. D. Starkie, of the Plastics Division of Imperial Chemical Industries, Ltd. Dr. Starkie demonstrated two prototype Schmidt television projection systems for home receivers, using cathode ray tubes of 2¼ or 3½ in. diameter, respectively. The picture size given by both systems is 16 in. × 13 in., the chromatic aberration is small, and the resolution is claimed to be far better than is required for the present B.B.C. television transmissions using 405-line scanning. Although the smaller of the two systems requires a 'throw distance' of 40 in. between the cathode ray tube and the viewing screen, it was shown how this could conveniently be incorporated in a receiver console of normal dimensions. A third Schmidt system suitable for television projection in small cinemas was also demonstrated: this contained a mirror of 18 in. diameter and gave a picture with a length of diagonal of 12½ ft.

Many more abstracts like these can be found in *A Bedside Nature: Genius and Eccentricity in Science, 1869–1953*, a 266-page book edited by Walter Gratzer. Contact David Plant, e-mail: subscriptions@nature.com

Asteroids

Made of craters

Mathilde is a very odd asteroid, for its surface turns out to be covered in giant craters. It is also exceptionally dark, reflecting just 4% of the light that falls on it, and exceptionally light, just 1.3 to 1.5 times the density of water.

The picture includes one of the largest craters, more than 30 km across (half the diameter of Mathilde). The image is from the NEAR (Near Earth Asteroid Rendezvous) spacecraft, which flew within 1,200 km of Mathilde on 27 June, en route to its main destination, an orbit around the near-Earth asteroid Eros. During the brief flyby, NEAR measured Mathilde's mass from its gravity. Its density is roughly half that of the meteorites that come from this class of asteroid, so presumably Mathilde is full of cavities.

Those huge craters must have been made by the impacts of asteroids several kilometres across, which should have broken Mathilde up. How has it survived?



The density measurement may be a clue: if Mathilde is half rock, half void, its structure might dampen the impact shock waves.

Another puzzle is the asteroid's slow rotation-period of once in 17.4 days. Those same impacts, mostly being off-centre, would have made it spin rapidly. It must have lost angular momentum somehow, perhaps by outgassing like a comet (but why so violently?), or perhaps by tidal dissipation from a large satellite (but where is that satellite now?).

Some questions are easier to answer. One of the principal investigators, Joe Veverka, was asked at a press conference whether the shadowed craters might be holes that go all the way through. "No," he said, "we don't see any stars."

Stephen Battersby

Familial Parkinson's disease

The awakening of α -synuclein

Michel Goedert

Parkinson's disease is characterized by a progressive neuronal degeneration which predominantly affects the dopaminergic nerve cells of the substantia nigra in the brain; the surviving cells contain characteristic cytoplasmic inclusions known as Lewy bodies, which are not normally present. Like many neurodegenerative disorders, Parkinson's disease either occurs in a common sporadic form or it can be inherited in a much rarer, familial form. But unlike other disorders, the nature of the mutations associated with familial Parkinson's were unknown. This has now changed with the discovery of a mutation in α -synuclein, a presynaptic protein, in four kindreds with early-onset, autosomal-dominantly inherited Parkinson's disease, reported last month in *Science* by Polymeropoulos *et al.*¹

Last year, Polymeropoulos and colleagues established linkage of familial Parkinson's disease (FPD) in a large, well-characterized Italian-American kindred to a region on the long arm of chromosome 4 (ref. 2). Their latest work has pinpointed the gene within this region that is mutated in FPD, namely that encoding α -synuclein. Moreover, the mutation has been identified as an alanine-to-threonine amino-acid substitution at position 53 (denoted as A53T) of this protein¹.

This same mutation was found not only in the Italian-American kindred but also in three apparently unrelated Greek families with FPD. The A53T substitution in α -synuclein segregated with the disease in all four pedigrees, with the exception of one individual from the Italian-American kindred, who had Parkinson's but had apparently inherited a different mutation from his father. None of 157 control individuals had the A53T change.

Synucleins are highly conserved, with rodent and zebrafish α -synucleins being respectively 95 and 86% identical to the human protein^{3–5}. One surprising feature is that the mouse, the rat and the zebrafish all have a threonine at position 53 of α -synuclein, like the mutant human protein. This raises the possibility that the A53T change could represent a rare polymorphism, although its presence in four unrelated kindreds with FPD makes this unlikely.

Human α -synuclein is an abundant 140-amino-acid protein of unknown function whose major site of expression is the nervous system, where it is concentrated in presynaptic nerve terminals^{3–7}. It was first identified in electric fish, and named synuclein in recognition of its apparent localization in presynaptic nerve terminals and portions of the nuclear envelope³. This nuclear localization was never confirmed,



Figure 1 Repeats in human α -synuclein.

Residues 7–87 of the 140-amino-acid protein are shown. Amino-acid identities between at least five of the seven repeats are indicated by black bars. The alanine $\rightarrow$ threonine mutation at residue 53 that is associated with familial Parkinson's disease is shown in the sequence between repeats four and five (A $\rightarrow$ T).

however, and all subsequent studies reported staining of only presynaptic nerve terminals.

Three different synuclein genes are known. Human α -synuclein has also been called NACP (for precursor of the non- β -amyloid component of Alzheimer's disease), because of the apparent association of one of its fragments with some of the extracellular β -amyloid deposits that constitute the neuropathology of Alzheimer's⁵. Human β -synuclein is 61% identical to α -synuclein and its gene maps to the long arm of chromosome 5 (refs 6, 8). Like α -synuclein, it is expressed at high levels in brain, where it localizes to presynaptic nerve terminals⁶. It also carries an alanine at position 53, suggesting that it might be worthwhile to look for mutations in β -synuclein in familial forms of Parkinson's disease. Unlike rodent and zebrafish α -synucleins, mouse, rat and bovine β -synucleins all have an alanine at position 53 (refs 9, 10). A third synuclein (synuclein-like protein) is mainly expressed in the peripheral nervous system¹¹.

Synucleins have a highly conserved amino-terminal repeat region, a hydrophobic middle section, and a less well-conserved, negatively charged carboxy-terminal region. They are modified post-translationally, but the exact modifications are not known⁶. Over half of the α -synuclein molecule is taken up by seven imperfect repeats of eleven amino acids, each having a conserved six-amino-acid core with the consensus sequence KTKEGV (in single-letter amino-acid code; see Fig. 1)^{3–5}. Core sequences of individual repeats are separated by five amino acids, with the exception of repeats four and five, which are separated by nine amino acids. The A53T change associated with FPD lies in this nine-amino-acid sequence (Fig. 1).

Although the function of the repeats is unknown, clusters of basic and acidic amino acids often mediate binding between structural proteins. Biophysical studies have shown that α -synuclein has no significant secondary structure¹². Such natively unfolded proteins frequently potentiate protein–protein interactions and become

structured when bound to other proteins¹². It will be important to identify binding partners for α -synuclein, as the A53T mutation may disrupt its normal function.

Proteins or fragments of proteins that are mutated in familial neurodegenerative disorders frequently accumulate as deposits that characterize the neuropathology of these disorders. Examples include some familial forms of Alzheimer's disease and some inherited prion diseases. It will no doubt be known soon whether or not α -synuclein is an intrinsic component of the core of the Lewy body.

Neurofilaments and ubiquitin can be detected in Lewy bodies^{13,14}, but it is unclear whether they are major components of the fibrillar and amorphous material of the core. Human recombinant α -synuclein has been shown to dimerize¹⁵, but it appears to be more soluble than the recombinant protein from the zebrafish, which aggregates in solution^{4,12}. Human α -synuclein with the A53T mutation might thus have an increased tendency to aggregate, especially if the mutation also interferes with the ability of α -synuclein to interact with its binding partner in nerve terminals. Rodent and zebrafish α -synucleins would not aggregate in nerve cells because they would be bound to other proteins and also because these species have a much shorter lifespan than humans. Lewy bodies are as much a feature of Parkinson's disease as is the dramatic loss of neuronal cells in the substantia nigra.

But Lewy bodies also occur in the brains of patients with other neurodegenerative disorders, especially in the cerebral cortex of patients with a late-life dementia that is clinically similar to Alzheimer's disease. The new work¹ brings α -synuclein to centre-stage, with the promise of new insight into the biology of the Lewy body and the pathogenesis of Parkinson's disease. □

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Daedalus

Guided lightning

Why does lightning go so far? A 100-MV spark will only bridge 20 metres or so; yet a thunder cloud at 100 MV can launch lightning flashes over 1 km long. This is because lightning travels in steps. A 'stepped leader' stroke descends about 20 m from the charged cloud, and pauses. The current ionizes the air along its track; it becomes so conducting that in a few microseconds its sharp end is raised to the 100-MV potential of its cloudy origin. A sharp point at a high potential, of course, is an ideal initiator of electric breakdown. So the stroke proceeds another 20 m, and recreates another sharp conducting point lower down, which breaks down the local air in its turn. Once begun, a stepped flash can travel indefinitely.

So, says Daedalus, with a 100-MV source we could launch our own lightning through any distance of air. But how to aim it? An ultraviolet laser has been used to ionize a path through low-pressure gas, to guide an electron beam; but air would absorb such a beam. These days, however, gyatron valves can generate brief 100-MW pulses at 150 GHz. So DREADCO plasma physicists, with some trepidation, are launching a pulsed gyatron beam from a sharp-ended hollow waveguide which has been charged to 100 MV. The intense narrow beam will energize the air along its track, so that the first 20-m lightning step will follow the beam. The skin effect of the 150-GHz modulation will preferentially ionize the outside of the discharge, forming a hollow waveguide carrying the bulk of the radiation to the end of the step. Here the process will repeat. At the next gyatron pulse, the discharge will step another 20-m step in the beam direction, and so on. Indeed, even a 5-MV source should work; its lightning would advance along the guiding beam in 1-m steps.

As a anti-aircraft weapon, DREADCO's lightning launcher could be quite humane. Most of its power will be dissipated along its track. The target will receive a shock strong enough to wreck its navigation and weapon electronics while causing little structural damage. The lightning launcher could also be useful in rain-making, by triggering potential thunderstorms — ideally from an aircraft above the clouds, to avoid receiving a prompt natural lightning blast back along the ionized channel. Religious zealots would also welcome an airborne lightning launcher. They could direct an apparent thunderbolt at York Minster or Canterbury Cathedral whenever a bishop uttered a heresy.

David Jones

Obituary

Dorothy Hill (1907–97)

Geologist with a passion for ancient corals, and the first woman to reach the pinnacles of Australian science



Dorothy Hill, who died on 24 April aged 89, maintained a remarkable association with the University of Queensland, in her native Brisbane, over a period of 70 years — in turn as student, teacher, administrator and benefactor. The scientific world will remember her as the twentieth century's authority on Palaeozoic corals; Australia will remember her as the country's most acclaimed woman scientist of her era.

As a first-year undergraduate she was influenced towards geology by the teaching of Professor H. C. Richards, who became her mentor. She chose to pursue palaeontology in particular because it was an acceptable profession for a woman in Australia in the 1920s, because there was work to be done and because it did not require expensive equipment. Her passion for corals came, not from the nearby Great Barrier Reef, but from a holiday encounter with a small Lower Carboniferous reef in the Wide Bay district of Queensland. In 1930 she won a scholarship to Cambridge University, and during the next seven years studied the Carboniferous corals of Scotland and Queensland and, more importantly, undertook an original review of coral morphology.

Coupled with a prodigious taxonomic and biostratigraphic evaluation of Australian fossil coral faunas during the period 1937–42, this work established Dorothy Hill as the pre-eminent worker in the field. Her research with W. H. Bryan on spherulitic crystallization in hexacorals

pioneered study of coral microstructure, which she introduced as a classification tool that remains widely used today; and her *Re-interpretation of the Australian Palaeozoic Record, Based on a Study of the Rugose Corals* of 1943 significantly advanced Australian stratigraphy, settling some long-standing disagreements on the ages of various strata.

After four years' war service as an officer in the Women's Royal Australian Naval Service, during which she worked on codes and cyphers, Dorothy Hill was invited by Ray Moore to contribute to the Anglo-American *Treatise on Invertebrate Paleontology*; this would occupy her for the next ten years and yielded the first comprehensive understanding of the Coelenterata. Concurrently, the Shell company began oil exploration in Queensland. Hill considered the Bowen Basin to be a good bet and began, with several postgraduate students, a study of the Permian brachiopods to establish a much-needed stratigraphy. Recognizing a lack of co-ordinated geological information for most of Queensland, together with Commonwealth and state geological surveys she became personally involved in fieldwork across the state to complete the reconnaissance mapping at 1:250,000; she also set out to compile a 40-mile-to-the-inch geological map and, with Alan Denmead, the chief government geologist, a volume on the geology of Queensland. These last two projects greatly influenced Australian geology, and resulted in a huge increase in mineral exploration activity for which Dorothy Hill helped train a stream of able young geologists.

Richards, her early mentor, had become president of the Great Barrier Reef Committee, and in 1946 he invited her to be the secretary of this body. In a 1942 paper, they had together described and interpreted two bores through the reef, and Richards had pushed strongly for a field station to be established on the reef at Heron Island. After his death in 1947, Dorothy Hill demonstrated her practical and administrative skills in supervising the erection and equipping of the station, which is still a major centre for reef research.

In the early 1960s she began a study of the extinct Cambrian phylum, *Archaeocyatha*, publishing on large Antarctic collections made by the British Trans-Antarctic Expedition and, in 1972, producing an authoritative and comprehensive volume on the group for the *Treatise on Invertebrate Paleontology*. During the early 1970s she began revision of Palaeozoic corals, of which known genera had doubled in 20 years mainly

from work in the Soviet Union and China. Her two revised volumes of the coral *Treatise* (1981) are not merely a compilation of the work of others, but the distillation of 50 years of her own intellectual enquiry. This zenith of her scientific achievements will remain the definitive treatment for some time.

As with so many people who lived through the Great Depression, Dorothy Hill found waste repugnant. Her students learned how to use a minimum of words and to fit the maximum number of specimens into a plate of fossil illustrations. She had no time for those who merely collected fossils, or even went further and studied and described them but did not contribute this knowledge to the literature. She set out to assemble a base of accurate knowledge for future generations to build on and she expected her students to share that aim.

In her own words Dorothy Hill was not a women's liberationist in the militant sense, believing that it is better to achieve by example rather than by force. What an example she set! She was the first woman to graduate from the University of Queensland with a gold medal, the highest accolade for an undergraduate; first woman president of the Royal Society of Queensland, 1949; first woman professor at an Australian university, 1959; first woman fellow of the Australian Academy of Science, 1956; first (and only) woman president of the Australian Academy of Science, 1970; first woman elected president of a professorial board at an Australian university, 1971; and first Australian woman fellow of the Royal Society, 1965. These 'firsts' are but a sprinkling of the many honours bestowed on her by government, scientific and cultural organizations in recognition of a career of sustained brilliance that was dedicated to science and public service.

Dorothy Hill lived a rich but quiet life, taking her greatest pleasure from overcoming intellectual challenges. Always gracious and patient, she had a ready wit and got on well with colleagues and students who in turn responded with commitment and loyalty. She rarely sought publicity and her name is not well known even in Australia. However, among scientists, the scale of her contributions is more widely understood — she inspired generations of them and encouraged many women into scientific careers. Her place among Australia's most eminent sons and daughters is assured.

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Fatigue, alcohol and performance impairment

Reduced opportunity for sleep and reduced sleep quality are frequently related to accidents involving shift-workers¹⁻³. Poor-quality sleep and inadequate recovery leads to increased fatigue, decreased alertness and impaired performance in a variety of cognitive psychomotor tests⁴. However, the risks associated with fatigue are not well quantified. Here we equate the performance impairment caused by fatigue with that due to alcohol intoxication, and show that moderate levels of fatigue produce higher levels of impairment than the proscribed level of alcohol intoxication.

Forty subjects participated in two counterbalanced experiments. In one they were kept awake for 28 hours (from 8:00 until 12:00 the following day), and in the other they were asked to consume 10–15 g alcohol at 30-min intervals from 8:00 until their mean blood alcohol concentration reached 0.10%. We measured cognitive psychomotor performance at half-hourly intervals using a computer-administered test of hand–eye coordination (an unpredictable tracking task). Results are expressed as a percentage

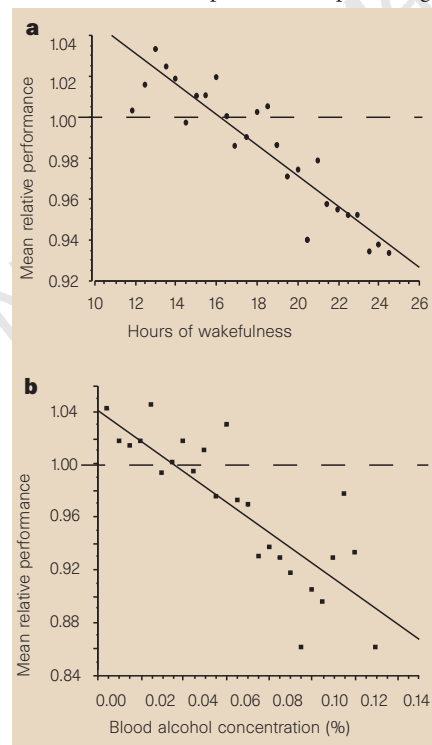


Figure 1 Scatter plot and linear regression of mean relative performance levels against: **a**, time, between the tenth and twenty-sixth hour of sustained wakefulness ($F_{1,24}=132.9$, $P<0.05$, $R^2=0.92$); and **b**, blood alcohol concentrations up to 0.13%, ($F_{1,24}=54.4$, $P<0.05$, $R^2=0.69$).

of performance at the start of the session.

Performance decreased significantly in both conditions. Between the tenth and twenty-sixth hours of wakefulness, mean relative performance on the tracking task decreased by 0.74% per hour. Regression analysis in the sustained wakefulness condition revealed a linear correlation between mean relative performance and hours of wakefulness that accounted for roughly 90% of the variance (Fig. 1a).

Regression analysis in the alcohol condition indicated a significant linear correlation between subject's mean blood alcohol concentration and mean relative performance that accounted for roughly 70% of the variance (Fig. 1b). For each 0.01% increase in blood alcohol, performance decreased by 1.16%. Thus, at a mean blood alcohol concentration of 0.10%, mean relative performance on the tracking task decreased, on average, by 11.6%.

Equating the two rates at which performance declined (percentage decline per hour of wakefulness and percentage decline with change in blood alcohol concentration), we calculated that the performance decrement for each hour of wakefulness between 10 and 26 hours was equivalent to the performance decrement observed with a 0.004% rise in blood alcohol concentration. Therefore, after 17 hours of sustained wakefulness (3:00) cognitive psychomotor performance decreased to a level equivalent to the performance impairment observed at a blood alcohol concentration of 0.05%. This is the proscribed level of alcohol intoxication in many western industrialized countries. After 24 hours of sustained wakefulness (8:00) cognitive psychomotor performance decreased to a level equivalent to the performance deficit observed at a blood alcohol concentration of roughly 0.10%.

Plotting mean relative performance and blood alcohol concentration 'equivalent' against hours of wakefulness (Fig. 2), it is clear that the effects of moderate sleep loss on performance are similar to moderate alcohol intoxication. As about 50% of shift-workers do not sleep on the day before the first night-shift⁵, and levels of fatigue on subsequent night-shifts can be even higher⁶, our data indicate that the performance impairment associated with shift-work could be even greater than reported here.

Our results underscore the fact that relatively moderate levels of fatigue impair performance to an extent equivalent to or greater than is currently acceptable for

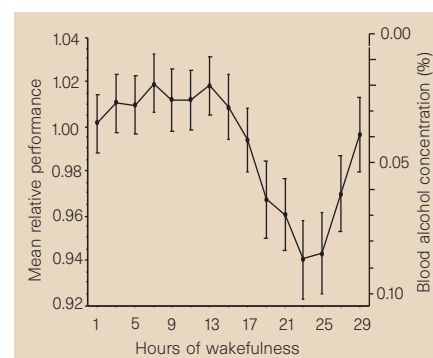


Figure 2 Performance in the sustained wakefulness condition expressed as mean relative performance and the percentage blood alcohol concentration equivalent. Error bars $\pm$ s.e.m.

alcohol intoxication. By expressing fatigue-related impairment as a 'blood-alcohol equivalent', we can provide policy-makers and the community with an easily grasped index of the relative impairment associated with fatigue.

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Entropy difference between crystal phases

In a recent Letter¹, Woodcock reported the results of a molecular dynamics study in which he claims to have finally determined the free-energy difference between the hexagonal close-packed (h.c.p.) and face-centred cubic (f.c.c.) phases of a crystal of (classical) hard spheres. Woodcock reports a small positive difference in the reduced Gibbs free-energy, which is equivalent to a difference in the reduced Helmholtz free-energy of $\Delta F^* \equiv (F_{\text{h.c.p.}} - F_{\text{f.c.c.}})/RT = 0.005(1)$ at the melting density (R is the gas constant, T is the absolute temperature, and the num-

ber in parentheses is the estimated error in the last digit). As Woodcock correctly points out, the calculation of the relative stability of the f.c.c. and h.c.p. phases of hard spheres is a long-standing problem in statistical physics. Attempts to resolve it date back to the work of Alder, Hoover and colleagues^{2–5}, and most recently, a direct simulation by Frenkel and Ladd⁶, obtaining the bounds of Helmholtz free-energy of $-0.001 \leq \Delta F \leq 0.002$. Woodcock's estimate is incompatible with this latter result.

To resolve this issue, we made accurate calculations of the free-energy difference between h.c.p. and f.c.c. hard-sphere crystals both at the melting density (73.6% of the density of regular close packing) and at close packing, using two different methods. We find that $\Delta F = 0.0009(2)$ at melting, a result that is quite consistent with the earlier work, but is five times smaller than Woodcock's estimate. Woodcock does not explain how he arrives at an error estimate of 20% — our work suggests that the numerical error in his result must have been four times larger than the entire h.c.p. – f.c.c. free-energy difference.

Nevertheless, we do agree with the sign of Woodcock's estimate — the f.c.c. crystal is indeed more stable than the h.c.p. crystal. This might explain the tendency towards f.c.c. packing seen in some experimental studies of hard-sphere colloids⁷. In one set of simulations, we used the 'Einstein-crystal' method^{6,8}, simulating crystals of 12,096 hard spheres (slightly larger than the largest system studied by Woodcock), and computed the Helmholtz free-energies of the two phases using a 20-point Gauss–Legendre quadrature. Every point in this quadrature involved a Monte Carlo simulation of 10^5 trial moves per particle, excluding equilibration. We find that the free-energy difference between h.c.p. and f.c.c. at melting is $\Delta F = 0.00087(20)$, and at close packing $\Delta F = 0.00094(30)$. The statistical error was computed on the basis of the variance in the block averages of the individual Monte Carlo runs⁹.

We also performed simulations using a new 'multi-hamiltonian' method (S.-C. M. and D. A. H., manuscript in preparation) that directly equilibrates the h.c.p. and f.c.c. hard-sphere crystals with each other by a set of intermediate states with different interactions but essentially the same free-energy. These latter simulations were done on much smaller samples (64 to 512 spheres) and obtained essentially the same free-energy differences (for 512 spheres, $\Delta F = 0.00085(10)$ near melting, and $0.0011(2)$ at close packing) as the 'Einstein-crystal' simulations, with comparable statistical errors. Statistically significant finite-size effects were detected only for the smallest size (64 spheres) near melting, where ΔF dropped to near zero.

In any event, our result for the f.c.c.–h.c.p. free-energy difference for large hard-sphere crystals at melting is much closer to $\Delta F = 0$, proposed almost 30 years ago by Alder and co-workers, than to the recent estimate by Woodcock.

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Woodcock replies — I reported the discovery a substantial area of pressure difference (ΔP) between the f.c.c. and h.c.p. single-occupancy-cell models, which arises from a difference in order–disorder transition pressures. The result was a free-energy difference in favour of f.c.c., corresponding to an entropy difference $0.005Nk_B$, over the range $V = 1.00N\sigma^3$ to $1.25N\sigma^3$, with a generous uncertainty (± 0.001), estimated by integrating the standard deviations of sub-averages of ΔP for individual data points. Extension of the computations on either side of the phase transition have since revealed a tail in the pressure difference for $V > 1.25N\sigma^3$ in favour of h.c.p. There is also a weak pressure difference for volumes below melting. I have now obtained more accurate data for these tails, including new data points on both sides of the single-occupancy-cell phase transition (Fig. 1).

I did not originally calculate the pressure difference in the stable crystal range, relying on earlier findings that ΔP up to melting was not detectable by molecular dynamics computation², and that these showed the two crystals to have indistinguishable crystal constants C_0 and C_1 (ref. 4). Consequently I assumed no difference between the Gibbs and Helmholtz free-energies in the stable

crystal range.

A detectable pressure difference between f.c.c. and h.c.p. crystals below melting, however, has now been computed, both by R. Speedy (personal communication) and myself. This small pressure difference means that the entropy difference at constant volume — which equals the Helmholtz free-energy difference for hard spheres — is not the same as the Gibbs free-energy difference, which determines the stable crystal structure at freezing. However, the correction is small, $\sim 0.000015Nk_B T$.

At the melting volume (V_m) of $0.96N\sigma^3$, I calculate the pressure difference to be $0.0030(5)k_B T/\sigma^3$ ($N = 12,000$). Alder *et al.*³ adopted too large a value for ΔP_m ($0.02k_B T/\sigma^3$), and further guessed wrongly that the absolute difference decreased linearly with density to zero at V_0 . In fact they estimated the Helmholtz free-energy difference ($\Delta F_m - \Delta F_0$) to be $0.002Nk_B T$ in favour of f.c.c. My data (Fig. 1) show that the pressure difference found at melting actually decreases to negligible values more rapidly, and that the change in free-energy difference between close packing and melting is of the order $0.0003Nk_B T$. The closeness of the result of Alder *et al.* to any of the present results, or indeed to zero, is therefore an irrelevance.

The Einstein-crystal method¹⁰ (used both by Frenkel and Ladd⁶ and here by Bolhuis and Frenkel), the multi-hamiltonian method and the Hoover–Ree single-occupancy-cell method, if accurately implemented, should all give the correct answer. I am still working on this problem, but the latest result for the Helmholtz free-energy difference between the h.c.p. and f.c.c. structures (f.c.c. having the lower free-energy) at close packing gives:

$$\Delta F_0 = \int_{V_0}^{\infty} (P_{\text{h.c.p.}} - P_{\text{f.c.c.}}) dV = 0.0026 \pm 0.001 Nk_B T.$$

The change in Helmholtz free-energy difference between close-packing and the melting volume amounts to only $0.0003(1)Nk_B T$, as shown by the tiny, positive area in $\Delta P(V)_T$ up to the melting volume (V_m) (see Fig. 1). Hence, the Helmholtz free-energy difference at the melting volume is $\Delta F_m = 0.0023(10)Nk_B T$. There remains a quantitative disagreement between my result and the other two methods, but my original conclusion that the f.c.c. phase is everywhere the more stable crystal phase for hard spheres is confirmed by all the new results. It is also gratifying that the result for the tiny free-energy difference between close packing and melting show a remarkable consistency, within the error bars, by all three methods.

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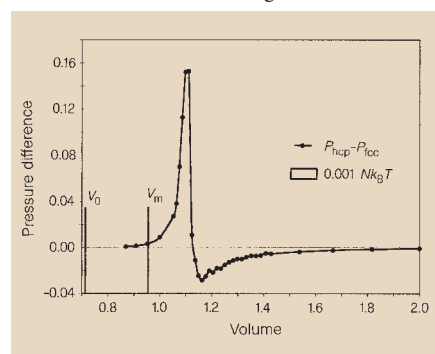


Figure 1 Latest molecular dynamic data for the pressure difference as a function of volume at constant temperature, $\Delta P(V)_T$, between the h.c.p. and f.c.c. single-occupancy-cell crystal structures for hard spheres; V_0 is the close-packed crystal volume and V_m is the volume at melting. The area under this curve is the Helmholtz free-energy difference between the two crystal structures at close packing in units of $Nk_B T$.

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Metallothionein in snail Cd and Cu metabolism

Terrestrial snails tolerate elevated concentrations of cadmium and copper, accumulating both metals in their soft tissues¹. The snails are able to inactivate the toxic cadmium while meeting their metabolic requirement for copper. Here we report evidence for the metabolic discrimination between the two metals based on the existence of distinct metallothionein isoforms, one dedicated to cadmium detoxification and another to copper regulation.

Even snails living in relatively unpolluted environments have the exceptional ability

to concentrate cadmium — more than many other terrestrial invertebrates — in the midgut gland². In contrast, copper, which is an essential constituent of the oxygen-carrying protein haemocyanin^{3,4}, is predominantly present in the snail's foot and mantle¹. The concentration of copper is kept constant, with animals quickly eliminating any excess that may have entered the tissue after environmental exposure¹. We have recently isolated and characterized two metallothionein isoforms from terrestrial helcid species, differentially involved in the handling of cadmium and copper.

One of these isoforms is present in the midgut gland of terrestrial snails. We identified it as a class-I metallothionein⁵ with a typically low molecular mass (6.62×10^6 ; 6,620K), containing 66 amino acids, 18 of which are cysteines. Its amino-terminal serine is acetylated (Fig. 1). This isoform occurs in several variants in helcid snails, including *Helix pomatia* and *Arianta arbustorum*^{6,7}.

The function of this isoform is the detoxification of cadmium, binding 85–95% of all cadmium accumulated in the snail soft tissues. The cadmium-binding metallothionein isoform can be isolated in a pure form from the midgut gland of metal-exposed snails, and has a molar metal ratio of Cd:Cu:Zn of 100:2:6.6 in the native protein and a stoichiometry of six cadmium atoms per protein molecule (determined by spectrophotometric metal titration under nitrogen atmosphere). Its concentration increases linearly with increasing cadmium concentrations in the midgut gland (Fig. 2a).

We have recently isolated another isoform from the mantle of *Helix pomatia*. Apart from its acetylated amino-terminal serine, the primary structure is very different to the cadmium-binding metallothionein. It has a different molecular mass (6,247K), and many amino-acids between the conserved cysteine residues have been substituted (Fig. 1). *In vivo*, this isoform is almost exclusively conjugated with copper, with a molar metal ratio of Cu:Cd:Zn of 100:1:6. We determined the stoichiometry using combined atomic absorption spectrophotometry, amino-acid analysis and electrospray mass spectrometry, as roughly six copper atoms per protein molecule.

The concentration of the mantle isoform and its exclusive preference for copper remain unaffected when snails are exposed to cadmium (Fig. 2b), even if this metal is injected into the mantle tissue. In this case, most of the administered cadmium is quickly eliminated from the mantle and redistributed to the midgut gland, but virtually none of the metal becomes bound to the copper-specific metallothionein isoform. In addition, the concentration of this isoform is barely affected by exposure of animals to large amounts of copper (Fig. 2b). Our results indicate that the metallothionein isoform in the mantle of terrestrial snails is concerned with the regulation of copper, probably in connection with haemocyanin synthesis (as the gastropod mantle is an important site of production of this copper-containing protein)⁸.

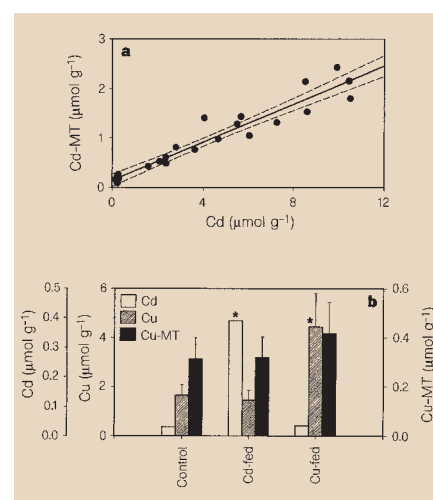


Figure 2 **a**, Linear relationship (bold line; regression coefficient $r = 0.96$), with 95% confidence limits (hatched lines) between molar concentrations (on a tissue dry-mass basis) of Cd and Cd-metallothionein (Cd-MT) in the midgut gland of *H. pomatia* fed on a Cd-enriched diet (3.5–955 μg Cd per g dry mass) for 14 days. **b**, Molar concentrations of Cd, Cu, and Cu-metallothionein (Cu-MT) in the mantle of *H. pomatia* after feeding the animals on uncontaminated salad (control) or on Cd-enriched (Cd-fed; 260 μg per g dry weight) or Cu-enriched diets (Cu-fed; 530 μg per g dry weight) for 14 days. Mean concentration ± s.d. ($n = 7$). Asterisks indicate significant differences ($P < 0.01$) from control values (Student's *t*-test). Concentrations of Cd-metallothionein and Cu-metallothionein were determined by modified Cd- and Cu-saturation assays⁹ (removing Cu from the holo-metallothionein with ammonium-tetrathiomolybdate). Similar results (not shown) were obtained after injecting Cd and Cu into mantle tissue.

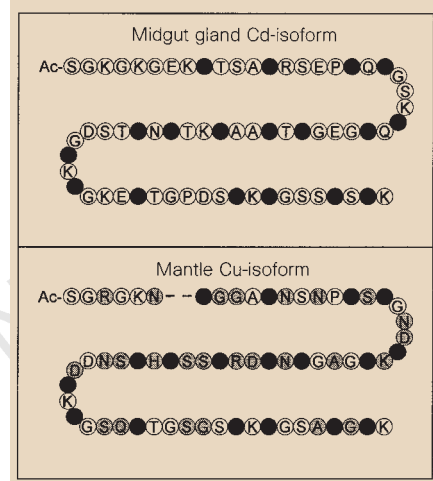


Figure 1 Primary structures of the cadmium- and copper-binding metallothionein isoforms from the midgut gland and mantle of *H. pomatia*. Residues are indicated using single-letter code, with cysteines in black. The N termini are acetylated (Ac). Substituted residues are indicated in grey in the copper-binding isoform. The cadmium-binding isoform was purified and sequenced as described earlier⁵. The copper-binding isoform was purified from mantle tissue by combined gel permeation, ion-exchange chromatography, and reversed-phase HPLC. After endoproteinase digestion (trypsin, Lys-C and Arg-C) of S-methylated protein, peptides were sequenced by collision-induced tandem mass spectrometry (API III, Sciex, Canada) using argon as the collision gas (4×10^{-4} molecules cm⁻²).

Until now, the simultaneous handling of different metals by metallothioneins has been explained on the basis of metal-specific preferences of the two metal-binding domains of the molecule^{9,10}. The existence of specific metallothionein isoforms dedicated to cadmium detoxification and copper regulation in snails suggests an alternative model to explain the mechanisms of multifunctionality in these proteins.

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A new SIV co-receptor, STRL33

The identification last year of chemokine receptors as fusion co-factors for HIV-1^{1–6} has contributed significantly towards understanding HIV transmission and AIDS pathogenesis (see ref. 7 for a review). Because the experimental infection of rhesus macaques with simian immunodeficiency virus (SIV) and the resulting development of an AIDS-like illness is the best animal model for HIV disease in humans⁸, identifying SIV co-receptors analogous to those used by HIV has obvious importance. We now report that STRL33, a chemokine receptor-like orphan receptor expressed in activated human lymphocytes and acting as a fusion co-factor with envelope glycoproteins (Envs) from HIV-1 strains of diverse

tropisms⁹, is a co-receptor for SIV.

The chemokine receptors CXCR4 and CCR5 are known to be important HIV-1 co-receptors for T-cell-line-tropic (TCL-tropic)¹ and macrophage-tropic (M-tropic) isolates^{2–6}, respectively. The chemokine receptors CCR2B and CCR3 can also act as co-receptors^{5,6}. Although CCR5, but not CXCR4, CCR1, CCR2B, CCR3 or CCR4, can serve as a co-receptor for diverse strains of SIV^{10–12}, it is also clear that human peripheral blood mononuclear cells express an SIV co-receptor(s) distinct from CCR5¹⁰.

First, we assessed SIV co-receptor activity of STRL33 and various chemokine receptors using the vaccinia-based cell fusion assay¹³. We tested cells expressing the Envs from SIV_{mac239} (TCL-tropic) and from SIV_{mac316} (M-tropic) for their ability to fuse with cells expressing CD4 and a single candidate co-receptor. STRL33 has potent fusion co-receptor activity with both SIV Envs (Fig. 1a). Consistent with previous reports^{10–12}, CCR5 functions with both Envs. We found no SIV_{mac} fusion co-receptor activity with CCR1, CCR2B, CCR3, CXCR4 (Fig. 1a) or CCR4 (not shown).

We next examined the ability of SIV_{mac239} to establish a productive infection in transfectants of human Jurkat T cells stably expressing STRL33 or CCR5. Both STRL33 and CCR5 render Jurkat cells permissive to SIV infection (Fig. 1b), whereas we observed no productive infection in the parental Jurkat cells (Fig. 1b) or in various transfectant control cell clones (data not shown). The basis for the difference in replication kinetics in the Jurkat-STRL33 and the Jurkat-CCR5 cultures is unknown — contributing

factors could be differences in the levels of co-receptor expression and the efficiencies of co-receptor function for SIV_{mac239}.

The discovery of individuals who are infected with HIV-1 despite being homozygous for an inactivating deletion in the CCR5 gene^{14–16} shows that at least one receptor other than CCR5 can be important in HIV disease. Further investigation of STRL33 and other members of the co-receptor repertoire is thus critical for understanding the natural disease process, and may assume added significance if HIV-1 is placed under selective pressure by therapies designed to block a specific co-receptor. Further, the activity of STRL33 with SIV_{mac} has relevance to human AIDS beyond the general parallels between the human and simian systems, as SIV_{mac} is phylogenetically close to, and thought to be the immediate progenitor of, HIV-2 (ref. 17), a virus known to cause AIDS.

Together with our previous report that STRL33 functions with HIV-1 strains of diverse tropisms⁹, our present findings with SIV demonstrate that STRL33 is active with a broader range of Envs than has been described for any of the co-receptors so far discovered. This attribute suggests that STRL33 will be of particular value in unravelling the structural determinants of interactions between Envs and co-receptors.

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•STRL33 is identical to Bonzo, one of the SIV co-receptors described by Deng *et al.* elsewhere in this issue¹⁸. See also N&V, pp. 230–231.

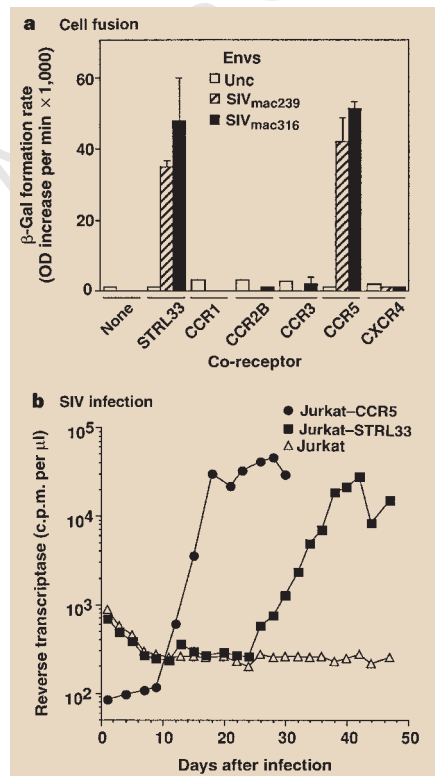


Figure 1 SIV co-receptor activity of STRL33, also referred to as Bonzo¹⁸. **a**, Vaccinia-based cell fusion assay (see citations in refs 1, 4 and 13 for methods). We transfected target cells (NIH 3T3) with plasmids containing the candidate co-receptor complementary DNAs linked to the bacteriophage T7 promoter and then co-infected them with vaccinia recombinant vTF7-3 encoding T7 RNA polymerase and vCB-3 encoding CD4. We co-infected effector cells (HeLa) with vCB-21R (containing the *Escherichia coli* LacZ gene linked to the T7 promoter) plus one of the following Env-encoding vaccinia recombinants: vCB-74 (SIV_{mac239}) or vCB-75 (SIV_{mac316}) (C. C. Broder and E. A. B., personal communication), or vCB-16 (nonfusogenic uncleaved Unc IIIb). We assessed cell fusion after 2 h by colorimetric assay of β-galactosidase activity in detergent cell lysates. **b**, Productive SIV_{mac239} infection of Jurkat cell transfectants. We prepared virus stock from HEK 293 cells transfected with SIV_{mac239} DNA and used it to infect Jurkat cell transfectants stably expressing STRL33 (ref. 9) or CCR5 (ref. 4) (as well as control Jurkat cells). We assessed virus production by measuring reverse transcriptase activity in the medium. The Jurkat-CCR5 infection was done in a separate experiment from other Jurkat infections.

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Tales of memory and imagination

Hystories: Hysterical Epidemics and Modern Culture

by Elaine Showalter
Columbia University Press/Picador: 1997.
Pp. 231. \$24.95, £16.99

Junk Psychology: Fallacies in Studies of 'Repression' and Childhood Trauma

by Harrison G. Pope, Jr
Social Issues Resources Series: 1997. Pp. 125.
\$12.95 (pbk)

Stuart Sutherland

Everyone loves stories or, as Elaine Showalter rather portentously calls them, "narratives". In *Hystories* — which might more appropriately, as she half admits, be entitled *Herstories* — she examines the tales developed by hysterics in both literature and real life.

She uses the term "hysteria" in the layman's sense to refer broadly to any hyper-emotional state accompanied by histrionic and attention-seeking behaviour. One of her rasher suggestions is that the British reaction to "mad-cow disease" was caused by the British fear of madness, a bizarre speculation given that the consumption of beef at one time declined more in Germany than in Britain.

Her review of the portrayal of hysteria in literature is not without interest, although her belief that it began with Flaubert's *Madame Bovary* and the Victorian romantics is odd. Perhaps she should try rereading Euripides' *Bacchae*.

Although she is a professor of English literature, her account of recent epidemics of hysteria in the United States is perhaps more worthwhile: although slight, it pulls together the different outbreaks, but the recent torrent of books on this subject makes it impossible to say anything new.

She deals with the syndromes of recovered memory, multiple personality, satanic ritual abuse and alien abduction, and courageously includes Gulf War and chronic fatigue syndromes as examples of hysteria: not only is it just possible that a pathogenic agent will be found in both illnesses, but the sufferers desperately want the cause to be organic, presumably to avoid the stigma of mental illness.

Like Freud, she concentrates on case histories rather than a scientific approach. She rightly stresses that people develop these syndromes partly as an excuse for and an explanation of their own failings and sometimes to escape punishment. Some have pleaded that they committed murder or rape as a different personality, and at least one person has pleaded in court that he had been forced to commit a crime by aliens.

Indeed, the most interesting aspect of

these "hysterias" is their causes, many of which Showalter does not mention. They include: the desire for attention; the comfort of being able to abdicate responsibility for one's failings by ascribing them to an illness; the luxury of receiving therapy (compounded in the case of multiple personality by the fact that in the United States therapy can now be obtained under health insurance thanks to its recognition as a disorder by the American Psychiatric Association); the excitement added to a humdrum life by weird beliefs about one's past; the propagation of hysterics' stories by the mass media; and the attempts by therapists to push their clients into revealing multiple personalities, non-existent sexual abuse, and even alien abduction in the mistaken belief that such revelation will effect a cure.

Finally, there may be a monetary pay-off for hysteria: although nobo 'y h. syet sued an alien abductor, people have obtained compensation from their fathers for abuse and others are busy suing the American military for the Gulf War syndrome. In a wry twist, patients are now prosecuting their therapists for breaking up their lives by instilling fictitious beliefs.

Showalter suggests that one cause of these hysterias is to give vent to repressed desires in concealed form. Women feel frustration for the sexual acts they have not performed and guilt for those they have. There is no suggestion that male hysteria, which is comparatively rare, is caused by sexual problems — although in this age men have plenty of those. She displays an uncritical acceptance of Freudian beliefs and ways of thought, an act of faith now virtually confined to novelists and literary critics, both professions more interested in a good 'narrative' than the truth.

The approach of Harrison Pope, a psychiatrist, could hardly be more different. He eschews all case histories on the grounds that they are biased by both the therapist's and the patient's ability to deceive themselves.

In evaluating whether there exist repressed but recoverable memories of traumatic events, Pope points out that in every study where survivors of a traumatic event, such as a collision between ships or the kidnapping of a school bus, have been interviewed several years later, all participants have had excellent recall — no sign of repression there.

He meticulously destroys the findings of a study by L. M. Williams which is the one most cited by believers in repressed memory: she interviewed 129 people who had been evaluated in hospital for signs of sexual abuse. Of these women, 49 claimed to have forgotten the abuse.



Using known facts on the reporting of abuse, Pope is able to show convincingly that their forgetting can readily be accounted for — at the time of the abuse some were under the age when memories can be formed; others had not been sexually abused at all according to the hospital's investigation; and a few did not want to disclose the abuse, a tendency well documented in other studies.

There is no need to invoke repression as the cause of forgetting: indeed there is no evidence for the existence of repression. Pope also demonstrates that the belief that sexual abuse propagates from one generation to the next is equally groundless.

Junk Psychology is a model of clear thinking and clear exposition. It outlines the pitfalls of epidemiology such as confounding causes: *post hoc* does not mean *propter hoc* — two correlated events may have a common cause, such as genetic factors.

To clarify his argument he analyses widely held but mistaken popular and medical myths: for example that salt is bad for you, that power lines damage the body, and that schizophrenia is caused by bad upbringing. Pope's careful analysis of possible sources of error should be useful to intending epidemiologists, and regrettably some practising ones, and to other disciplines within the social sciences.

A comparison of the two texts shows that, as is usually the case, the prose style and clarity of the scientist are far superior to those of the professor of English. In this postmodernist world, literary criticism might well fare better if it were taken over by scientists. But God help science if literary critics reciprocated this gesture. □

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Inside knowledge

Interpreting Minds: The Evolution of a Practice

by Radu Bogdan

MIT Press: 1997. Pp. 304. \$35, £29.50

Andrew Whiten

For most of us, reading others' minds is an everyday accomplishment: not only may we be concerned with what others *think* of us, but our thoughts may even extend to what *they* think *we* think of them. How and what we may know of other minds has long exercised philosophers, but the past decade has shown that our everyday mind-reading can be examined scientifically.

The results have been most prominent in the field of developmental psychology, which has seen the growth of a gigantic literature on what has come to be called the child's 'theory of mind'. Other researchers have attempted to reconstruct the evolution of such abilities by studying primates, following the experiments pioneered by David Premack and Guy Woodruff to discover whether the chimpanzee has a theory of mind.

It is not surprising that philosophers have

found this work of interest. What is more, there has recently been real dialogue between philosophers of mind and experimentalists, seriously enriching the scope of the scientific enterprise. Indeed, some philosophers have become so excited as to begin experimenting themselves. The philosopher Radu Bogdan is not one of these, but he has done an impressive job of assimilating the new empirical literature in all its breadth, and his book is a work of psychology as much as philosophy.

In his own words, his task has been to "get to the roots" of this extraordinary competence of ours. He pursues this in part by charting in considerable detail accomplishments identified from infancy to middle childhood, together with their hypothesized functions and underlying cognitive procedures.

This is not a book to read as an introduction to the field: it will be challenging even for those already familiar with the primary literature. But for the growing 'theory of mind' community, it provides a distinctive analysis of the most fundamental questions about how we interpret the actions of others.

This is important because, despite the outpourings of experimental results, the field still lacks any deep appreciation of the nature of

what 'mind-reading' can be, given the nature of mind: we are not telepathists, so what on earth can be going on when we act as if we are?

Bogdan's unique contribution is timely and welcome: it extends the dialogue between philosophers and cognitive scientists beyond recent constrained debates about whether 'theory of mind' is really theory-like or whether it operates by using the self's mind to simulate others; and, no less important, it goes beyond taking evolutionary psychology seriously, to put it at the heart of the analysis.

But what Bogdan has to say can also be problematic. At many points I found the text impenetrable, over-abstract or unconvincing in its conclusions; as a margin-scribbler, I have seldom added so many question marks, including those of the "meaning what?", "who says?" and "how do we know?" variety.

Bogdan argues, correctly I think, that the practical use of mind-reading has not been adequately addressed in developmental psychology. But his argument — that recognizing the evolutionary history of its practical benefits reduces the need to explain the abilities of natural psychologists as mind-reading (he prefers to talk only of "interpretation") — is insufficiently clear or persuasive. I do not see practical use and the recognition of states of mind as being in opposition; and evolutionary theorists have neglected neither.

Nevertheless, Bogdan's analysis should provoke thoughtful consideration and discussion across philosophy, cognitive science and ethology, in which the nature of mind-reading is treated with a much-needed new depth and breadth. □

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Picnic in the park

Visitors have been photographing Yellowstone National Park in Wyoming since its earliest days. The early tourists pictured here are on a picnic in the Upper Geyser Basin in about 1880. This is

one of nearly 400 photographs featured in *A Yellowstone Album* (Roberts Rinehart, \$19.95), which commemorates the park's 125th anniversary.

Ways of seeing

Art and Representation: New Principles in the Analysis of Pictures

by John Willats

Princeton University Press: 1997. Pp. 394. \$39.50, £26.95

John M. Kennedy

John Willats argues that picture perception can be broader in scope than real-world perception. Take outline drawings, where a line typically shows the edge of a surface of an object. Curiously, the line usually has two borders yet they show only one edge: that is, the number of features of the line on the picture surface is not the number of features in the depicted scene.

Also, the features of the line can have more than one function. We may, for instance, draw a line around a patch of the picture surface merely to mean "this patch

stands for a facet of an object". Willats describes this as using the "region" on the surface to stand for a face of the referent.

What's more, we sometimes use the region to stand for the whole object — as a "volume". If someone making a line sketch wants us to take the region to be relevant, the line serves only as a convenient device to delimit the region — it is not meant to stand for an edge. Similarly, a Chinese brush painting of a furry animal may have a region of ink standing for the animal's body, but the edges of the inked patch are not to be taken as a detailed rendition of the animal's contours. And a line around the patch can be dotted like an outline in a mosaic from Ravenna.

On a misty morning, or after a celebration, we may often have seen a real edge of an object such as Tony Blair as slightly blurred, but we have never seen it as really composed of dots. Pictorial elements are more varied than real-world elements.

Willats contends that children beginning to draw can probably produce an elongated line and an oval shape. With these two picture-primitives, they can successfully draw only things extended mostly in one dimension (arms, stems, people, bottles) or roughly equally in three dimensions (houses, cars, elephants). Relying on this 'extendedness', young children have trouble drawing objects intermediate in shape between rocks and stems — ones extended in two dimensions mostly, such as 'slabs' like books, doors, plates and coins.

When we become proficient at drawing, particular junctions of lines have particular meanings. An L-junction can show the sharp, rectangular corner of a book. A T-junction, usefully, can show one surface edge overlapping another. The cross-bar of the T shows the foreground edge and the stem shows the edge of the rear surface. And the end of a line can show a fold in a surface diminishing to nothing.

As well as outline drawings, Willats describes the picture-primitives of rounded forms (in pictures by Paul Klee), silhouettes (used in early Greek vases) and crosshatchings of various kinds, including ones where shading is used to suggest light and shadow (in nineteenth-century technical drawings of machinery, for example) and ones where the orientations of the hatches indicate the orientation of the depicted surface ('bracelet' shading, used in sixteenth-century engravings, for example).

Real objects do not come crosshatched, so once again picture perception cannot be predicted directly from real-world perception.

We often draw long objects such as nearby buildings using parallels to show the receding sides, even though the actual sides of the buildings make a smaller and smaller angle at our eye with distance. We find these

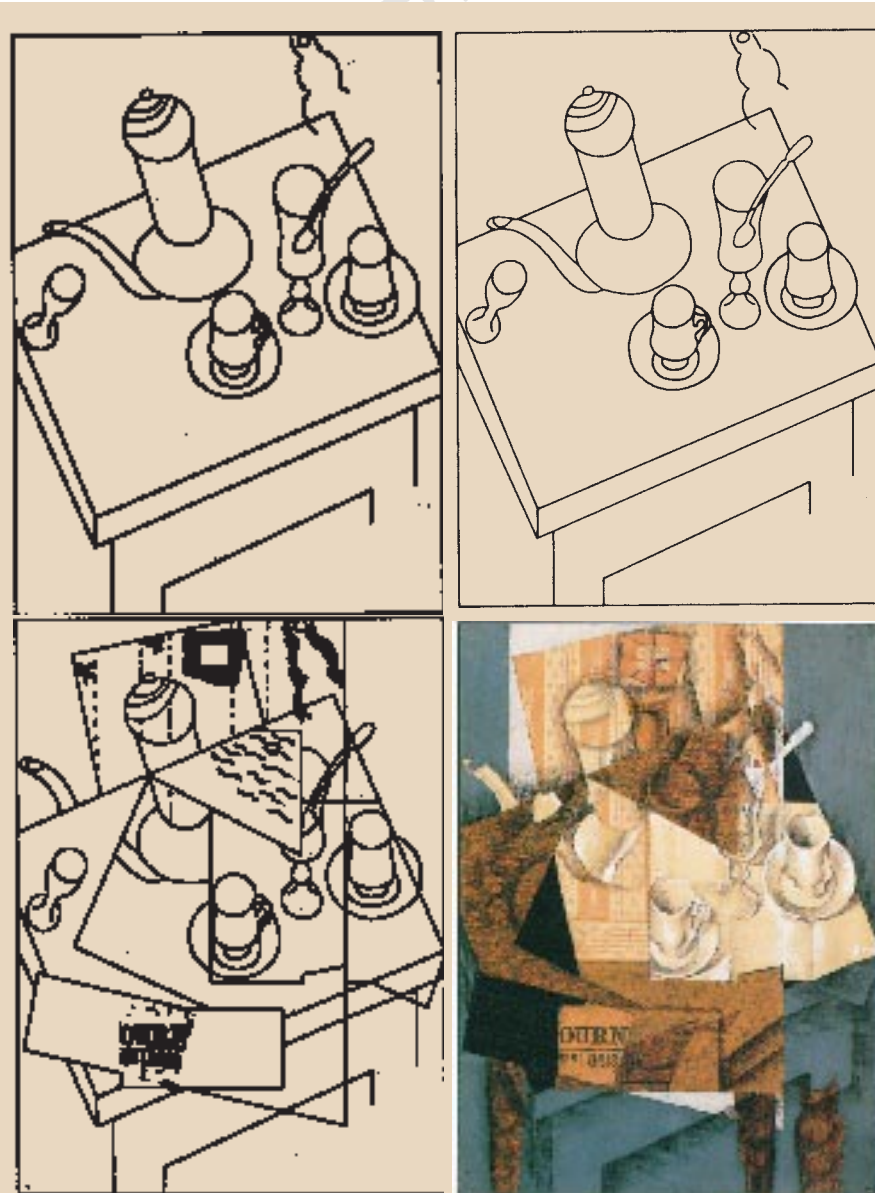
parallel projections quite acceptable even if the picture's projection at our eye is a far cry from nature's projection.

But we have limits to the range of projections that appear quite acceptable. The religious art of the Byzantine empire took advantage of vision's limits, artfully relying on divergent perspective — diverging lines on the picture standing for receding parallel sides of artefacts — to create anomalies. Willats stresses that these were meant to strike the Orthodox viewer as unrealistic — meant deliberately to be incoherent and self-contradictory.

In practice, we often make use of varied geometries of visual representation, exploiting the wide capacities of the visual system. Children do not simply draw by copying the shapes of objects as if making an imprint of a face of the object.

Willats explains that the child uses various geometries in the development of drawing prowess. A three-year-old is concerned with what is a detached object, what is enclosed in what and what touches what. These are features of topology, the 'rubber-sheet' geometry in which connections are preserved. The child attending to topological features will draw what is inside an object in the real world inside the region in the picture that stands for that object.

Only later in development will the child begin to copy the relevant shape of the object, so a square will stand for a cube. This is a shape produced by a similarity geometry, or orthogonal projection. But, Willats strikingly claims, at this stage the square stands for the entire cube. His evidence is that, if we give the child a cube with differently coloured faces, the child will colour



Making a meal of it: illustration from the cover of *Art and Representation*, showing the application of Willats's analysis to *Breakfast* by Juan Gris (1914; in the Museum of Modern Art, New York).

the square with differently coloured patches, one for each of the cube's faces.

I hesitate to accept his argument here, since the child may mean "this is a face of the object, and here are some colours it can have". I also balk at his proposal that the child makes much use of topology, since an X and an I are the same in topology, neither one enclosing a space, but the two are distinct for kindergarten children. Willats admits that children must add some "shape modifiers" to topology.

Willats points out that children often produce pictures that have traditionally been described as using one primary geometry of depiction — parallel projection that projects faces of the object onto the picture surface. If the parallel lines of the projection are at an oblique to the picture surface, side faces can be projected as well as front faces.

But, he argues interestingly, the child actually arrives at these drawings by a short circuit—a 'secondary' geometry—and not by imagining the full procedures of parallel or polar projection — 'primary' geometry of projection.

He writes that many of the puzzling shapes along the route of development "can be explained by supposing that they are derived from internal object-centred descriptions by the application of rules based on secondary rather than primary geometry".

A secondary-geometry rule the typical child uses to produce folded-out forms is "draw receding sides of objects attached to the front face, and with their true shapes". To suggest depth, the child may draw only the front face and one receding face.

A slightly more sophisticated rule in secondary geometry is "show the receding sides of a building by parallel lines at an acute angle to the horizontal". This rule allows the child to draw the front, the top and one side of an object — all the parts that can face a single vantage point. An advanced secondary-geometry rule is "make the lines for a roof's edges, receding into the distance, slope downwards in the picture" and "make the lines for any steps at the base of a building, also receding into the distance, slope upwards".

Willats argues that many pictures from classical times and thirteenth- and fourteenth-century Italy obey these rules about slopes. The rule many children eventually come to is "if the side recedes, draw it using converging lines".

In sum, Willats offers clear applications of his ideas to an immense diversity of pictures from various sources. His *tour de force* will be the jumping-off point for most picture-perception researchers in the near future. But what are those jumping-off points? The contentious issues can be divided into two camps, developmental and cross-cultural.

Willats' theory provides the child aged 6 to 11 with secondary geometry, deriving pictures from object-centred knowledge and rules about receding faces, but it leaves no clear place for perspective effects such as foreshortening. This allows Willats to predict that the child will transform receding shapes such as squares into diamonds or rhombic forms. But he cannot predict consistent foreshortening and in particular the actual amount of foreshortening the child will use.

Western children of 8, 9 and 10 do use foreshortening, reliably, and in particular they foreshorten receding sides of cubes to about 70 per cent of the size of the lines standing for the front edges of the cube.

The same limitation obtains when Willats examines the widespread use of oblique projection in pictures from China and Japan over the centuries. For example, the receding sides of screens depicted in a twelfth-century Chinese picture are also foreshortened about 70 per cent. It is likely that view-centred processes for making pictures are indicated by the 70 per cent foreshortening we see in these pictures from diverse sources. I suggest we need a theory of visual inspection, not secondary geometry, to explain why foreshortening crops up so regularly.

Willats' book bids fair to make the study of 'pictorial cues' central to vision science. It will shape debate about drawing development for many years. □

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Metallurgy – and other animals

Stuff: The Materials the World is Made of

by Ivan Amato

BasicBooks: 1997. Pp 304. \$25

Paul Calvert

Metallurgy used to refer simply to the extraction of metals, but the development of new alloys and of the electron microscope led to a science of the structure of metals. Metallurgy remains a proper discipline, with fundamental theories, methods and boundaries.

Things fell apart when the subject extended to become materials science, with the growing use of polymers, ceramics, glasses and composites in engineering. The problem is that all materials are different and we no longer have a discipline.

Ivan Amato does an excellent job of surveying the field without getting trapped

into improvising structure where there is none. He starts historically and takes us from flint-knapping to the formation of the first materials laboratories and departments in the 1960s.

He then draws on his experience as a science journalist visiting the US Materials Research Society (MRS) meetings. He makes an analogy with the Serengeti National Park in Tanzania, in the sense that this large area is populated by small herds of scientists, grazing together and wandering across the conceptual landscape. Since this prompts one to identify the rhinos, ostriches, hyenas and elephants among one's colleagues, the book is worth the price for this analogy alone.

The particular herds that Amato selects — he politely refers to them as tribes — are synthetic diamond, biomimetic materials, smart materials, fullerenes and gallium arsenide.

Two final chapters focus on particular stories: Federico Capasso at Lucent Technologies and the quantum cascade laser, followed by Greg Olson at Northwestern University and steel microstructures. This gives us a view of the individual antelope in the herd.

Most materials departments are called Materials Science and Engineering and sit in engineering colleges. But there are very few true materials engineers who actually design a system to do a particular job in a defined framework. Most work in research-and-development laboratories and cover the range from study-and-explain to enlightened trial-and-error.

So this is not a science as physics is, and it is not engineering as chemical engineering is. If there is a parallel science, it is biology in that it involves the study, and now engineering, of a collection of things that happen to exist.

The MRS, founded in 1973, reflects the Serengeti analogy. It is large but was designed to be fairly unstructured so it could respond to, rather than guide, the field. It has steered away from qualifications, accreditation and representation.

An interesting omission from *Stuff* is any reference to teaching. Most university materials laboratories have many undergraduate research assistants. The national laboratories carry out a large fraction of materials research and have a large population of graduate students borrowed from universities. I suspect we believe more in apprenticeship than in book-learning.

Materials science is now grown up. It may settle down and become a discipline or continue in fruitful anarchy. *Stuff* gives a good portrait of its youth. □

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A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade

Susan Tsunoda, Jimena Sierralta, Yumei Sun, Ruth Bodner, Emiko Suzuki*, Ann Becker, Michael Socolich & Charles S. Zuker

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How are signalling molecules organized into different pathways within the same cell? In *Drosophila*, the *inaD* gene encodes a protein consisting of five PDZ domains which serves as a scaffold to assemble different components of the phototransduction cascade, including the principal light-activated ion channels, the effector phospholipase C- β and protein kinase C. Null *inaD* mutants have a dramatically reorganized subcellular distribution of signalling molecules, and a total loss of transduction complexes. Also, mutants defective in a single PDZ domain produce signalling complexes that lack the target protein and display corresponding defects in their physiology. A picture emerges of a highly organized unit of signalling, a 'transducisome', with PDZ domains functioning as key elements in the organization of transduction complexes *in vivo*.

The cellular responses to a wide variety of external signals are mediated by plasma-membrane receptors that transduce extracellular stimuli into an intracellular response. Although receptors that recognize different ligands are known to interact with the same intracellular signalling molecules, the specificity of signalling, which is often essential to a cell's physiological role, is maintained. But how is this specificity maintained, or rather, how is signal cross-talk avoided? One solution may be that different signalling cascades are organized into physically and functionally distinct signalling units. Response time, specificity and selectivity would then be greatly enhanced, and cross-talk would be minimized. Although we know much about the function and regulation of many signalling pathways, little is known about the architectural organization of the corresponding machinery.

Many of the molecules involved in signalling contain small protein-protein interaction domains that may allow recruitment and assembly into larger complexes. These include the Src-homology SH2 and SH3 domains^{1,2}, pleckstrin-homology (PH)^{3,4} and phosphotyrosine-binding (PTB)⁵⁻⁷ domains, and postsynaptic density protein, disc-large, zo-1 (PDZ) domains⁸⁻¹². Many of the proteins interacting with, or containing, PDZ domains are localized at the plasma membrane¹³⁻¹⁵, so PDZ domains may provide a framework for the recruitment of target molecules into membrane-bound macromolecular complexes. Recently, the PDZ-domain postsynaptic density protein PSD-95 has been shown to mediate the clustering of both NMDA (N-methyl-D-aspartate) receptors^{12,16-18} and K⁺ channels¹⁹⁻²². Furthermore, members of the PSD-95/93 family have been implicated in the clustering of synaptic complexes^{16,23-25}, and a PDZ domain in the protein tyrosine-phosphatase Fap1 binds to the Fas membrane receptor²⁶. Also, PDZ-PDZ interactions could be important mediators of protein-protein interactions²⁶. So far, PDZ domains have been found in more than 50 proteins, including many involved in cell signalling²⁷⁻²⁹, although little is known about the function of PDZ domains *in vivo*.

The *Drosophila* phototransduction cascade is a G-protein-coupled, phospholipase C (PLC) signalling pathway that shares many features with other signalling cascades³⁰. *Drosophila* photoreceptor neurons also show a high degree of architectural organization, with most of the molecules involved in phototransduction

localized to the rhabdomeres, a specialized subcellular compartment consisting of approximately 60,000 tightly packed microvilli, each 1–2 μm in length and 50 nm wide³¹. In rhabdomeres, light activation of rhodopsin activates a G_q protein α -subunit (G_{q α}), which in turn activates PLC- β ³². PLC- β catalyses the breakdown of phosphatidylinositol-4,5-bisphosphate (PtdInsP₂) into the two intracellular messengers inositol trisphosphate (InsP₃) and diacylglycerol (DAG), leading to the eventual opening (and modulation) of the transient receptor potential (TRP) and TRPL light-activated channels³³. Following termination of the stimulus, calcium-dependent regulatory processes, including activation of protein kinase C (eye-PKC), mediate deactivation of the light response³⁴. The *inaD* (inactivation no-after potential) locus is one of many loci identified in genetic screens designed to investigate this signalling cascade. A single mutant allele, *inaD*²¹⁵, was isolated and shown to have a dominant-negative phenotype on photoreceptor deactivation^{35,36}. Previous studies showed that the InaD protein contains at least two PDZ domains³⁶ and interacts with the TRP ion channel³⁷. InaD was also reported to associate with multiple components of the phototransduction cascade, including PLC- β , eye-PKC, rhodopsin and calmodulin³⁷⁻³⁹. Thus it has been proposed that InaD is a regulatory component involved in feedback regulation of the light response³⁹. To determine the role of the PDZ-containing InaD protein *in vivo*, we sought to identify its interacting component(s), and to perform a genetic and physiological analysis of its function. We show that the InaD protein is composed of five distinct PDZ domains and acts as the organizing scaffold for photoreceptor signalling complexes *in vivo*. Our genetic, physiological and cell-biological studies demonstrate the presence of a multivalent adapter protein that links multiple signalling components within the same cascade. Our results also demonstrate that PDZ domains have a fundamental role in the assembly of transduction complexes *in vivo*.

InaD protein consists of five PDZ domains

InaD has been shown to associate with components of the phototransduction cascade³⁷⁻³⁹. Given the ability of PDZ domains to mediate protein-protein interactions, we sought to determine whether InaD is involved in the assembly of transduction complexes. We first defined the interaction between InaD and the

interacting proteins, and then screened for *Drosophila* mutants defective in this molecule. When we analysed the primary structure of InaD in detail³⁶, we found it to be a modular protein composed of five closely linked PDZ domains¹³ (Fig. 1). Each domain contains the structural characteristics of a prototypical PDZ motif, including the conservation of residues thought to be important for target binding²⁷. However, the five domains display enough variability between them, and with other members of the PDZ domain family, to allow distinct protein–protein interactions⁴⁰.

Interaction with the phototransduction cascade

Because InaD contains five distinct PDZ domains, we reasoned that it might interact with as many as five different components of the phototransduction cascade. We used immunoprecipitations and glutathione S-transferase (GST)–InaD fusion proteins to identify InaD targets. InaD antibodies co-immunoprecipitate TRP, eye-PKC and PLC- β from head extracts³⁸ (Fig. 2a), but not rhodopsin or G_{α} , despite the fact that both of these are extremely abundant in photoreceptor cells; identical results were obtained *in vitro* using a full-length GST–InaD fusion protein (Fig. 2b).

To identify the site on InaD that interacts with TRP, eye-PKC and PLC- β , we produced individual PDZ domains of InaD as GST fusion proteins and assayed them for interaction with each of these target proteins in whole head extracts (Fig. 2b). The third PDZ

domain of InaD is specific for TRP³⁷, the fourth domain interacts with eye-PKC, and the fifth domain interacts with PLC- β . Given the modular organization of PDZ domains in InaD, we next examined if binding to one target relies on the binding to the others. We found that immunoprecipitation of InaD from *trp* mutants, PLC- β null mutants (*norpA*) or PKC null mutants (*inaC*) still co-precipitates the remaining two targets (Fig. 2a). These results demonstrate some important aspects of the function of InaD and PDZ domains: different PDZ domains have different and highly specific targets; InaD functions as a modular multivalent PDZ protein interacting with different components of the same pathway; and these interactions are not formed by interdependency between the different partners.

inaD mutants do not form transduction complexes

If InaD functions *in vivo* as a scaffold to localize or assemble components of the phototransduction cascade into transduction complexes, then a null *inaD* mutant should display a total loss of signalling complexes and a redistribution of individual signalling molecules. Unfortunately, only a single *inaD* mutant allele had been isolated and this behaved genetically and physiologically as a partly dominant-negative mutation^{35,36}. We therefore tried to isolate new *inaD* alleles. We used a screening strategy based on the loss of InaD antigen on immunoblots, rather than on a hypothetical

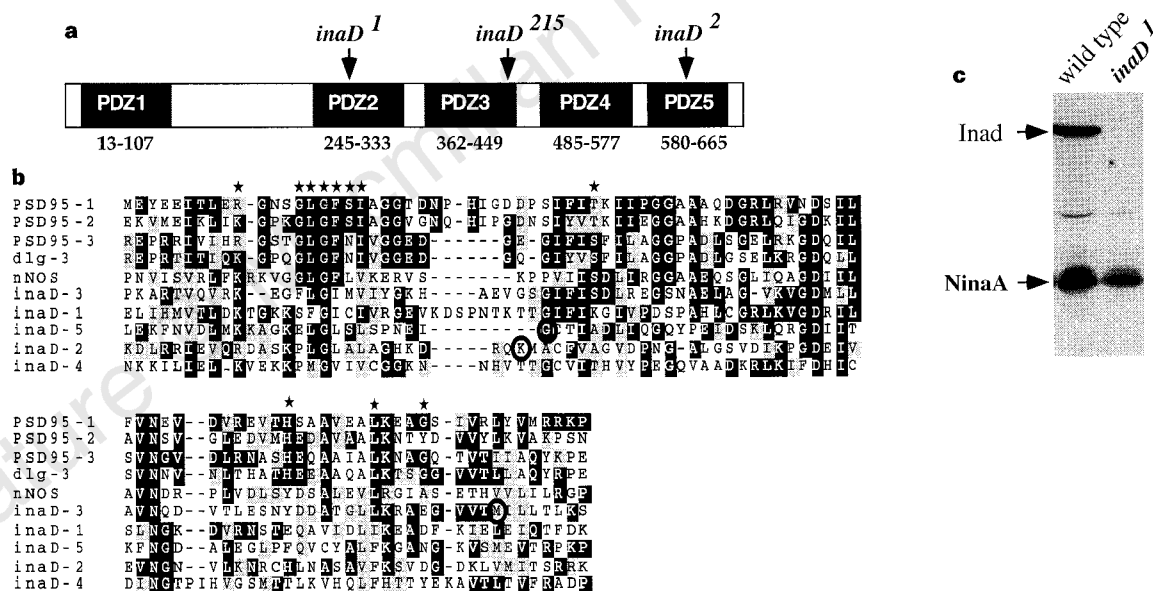


Figure 1 InaD is composed of five distinct PDZ domains. **a**, Diagram of the InaD protein, highlighting the location and size of each PDZ domain; also shown is the relative location of the three *inaD* mutations. **b**, Amino-acid alignment of PDZ domains from mammalian PSD-95 (ref. 9), nNOS (ref. 23), *Drosophila* Dlg (ref. 8) and InaD (ref. 36). Sequences were aligned to maximize similarities. Black boxes

indicate amino-acid identities, and grey boxes show conservative substitutions. Stars above the sequence indicate residues implicated in substrate binding²⁷. The circles residues refer to the site of point mutation in the three *Drosophila* *inaD* alleles (see text for details). **c**, Immunoblot showing the absence of InaD protein in *inaD*¹. NinaA was used as a control.

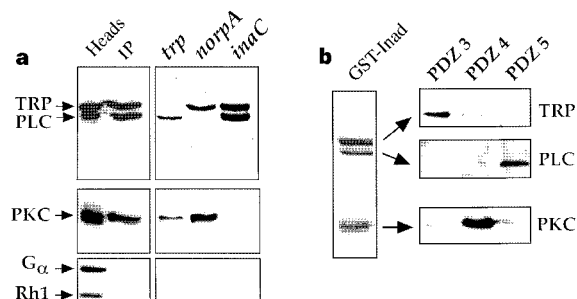


Figure 2 InaD forms a complex with PLC/PKC/TRP. **a**, Membranes prepared from the heads of wild-type flies (IP) or *trp*, *norpA* and *inaC* mutants were immunoprecipitated (100 μ g) with anti-InaD antibody. The immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific for TRP, PLC, eye-PKC, and rhodopsin (Rh1). "Heads" refers to membranes before immunoprecipitation. InaD co-precipitates with TRP, PLC and PKC, but not with Rh1 or G_{α} . As expected, immunoprecipitations from *inaD* nulls did not precipitate TRP, PKC or PLC (data not shown). **b**, A full-length GST–InaD fusion protein associates with TRP, PLC and PKC; overexpression of each of the individual PDZ domains from InaD as GST–PDZ fusions produce highly preferred interactions.

physiological or behavioural basis. We generated fly stocks containing individual homozygous mutagenized second chromosomes (*inaD* maps to the second chromosome at position 591B1-2), and each stock was then subjected to immunoblot analysis for the loss of anti-InaD immunoreactivity³³. Analysis of 2,847 lines yielded two alleles, *inaD*¹ and *inaD*²:*inaD*¹ has a complete loss of the protein, whereas *inaD*² expresses reduced levels of protein.

By using the polymerase chain reaction (PCR), we isolated the *inaD* gene from each mutant allele and determined the entire nucleotide sequence. We found that *inaD*¹ has an amber nonsense mutation at position 811, leading to premature termination of the polypeptide chain at amino-acid residue 270; this represents a complete null allele. The *inaD*² allele, however, has an A to G change at nucleotide 1814, leading to a substitution of a conserved glycine for glutamic acid in the fifth PDZ domain (Fig. 1).

We used immunofluorescent staining of frozen tissue sections to test for the subcellular localization of signalling molecules in the *inaD*¹ null mutant cells. TRP, PLC-β and eye-PKC are completely mislocalized in the *inaD*¹ mutant (Fig. 3). These proteins no longer localize to the rhabdomeres, but instead are found randomly distributed throughout either the plasma membrane (in the case of TRP) or the cytoplasm (PLC-β and eye-PKC). In contrast, rhodopsin, G_{qα} and TRPL distribute normally in *inaD*¹ (Fig. 3). Because signalling complexes are not formed in *inaD*¹ mutants, photoreceptors have profound signalling defects, responding only at the highest light intensities and with dramatically altered kinetics (Fig. 4).

We suspected that a loss of the scaffold and mislocalization of the signalling proteins may result in the instability of these target proteins. We therefore examined the steady-state levels of transduction proteins by immunoblot analysis at different stages post-eclosion. As predicted, the levels of TRP, PLC-β and eye-PKC are all markedly reduced in the *inaD*¹ mutants, and by 10 days post-eclosion are less than 10% of wild-type levels (Fig. 5). In contrast, the levels of rhodopsin, G_{qα} and TRPL are unaffected. Taken together, these results demonstrate that InaD has an important role in organizing signalling complexes, and show that these complexes are required for the stability of the target proteins.

Effects of mutations in PDZ domains

We have shown that InaD interacts with multiple components of the phototransduction cascade and is essential for the assembly of

signalling complexes. We reasoned that it should be possible to mutate the PDZ domain for a particular target and prevent the recruitment of that protein into the transduction complexes. This should generate *in vivo* phenotypes that resemble mutations in the target proteins.

The original *inaD* allele, *inaD*²¹⁵, has a missense mutation in the third PDZ domain³⁶. Because this domain is involved in the interaction of InaD with TRP (Fig. 2), and this mutation abolishes the interaction of TRP with InaD^{37,39}, we sought to determine whether the stability of TRP, its subcellular localization and its function are disrupted in *inaD*²¹⁵ mutants. We examined TRP protein levels by immunoblot analysis, its subcellular localization by immunofluorescent staining of tissue sections, and its function by performing whole-cell voltage-clamp recordings and electroretinograms.

We found that TRP protein levels decline with age in *inaD*²¹⁵ mutants (Fig. 5). In young *inaD*²¹⁵ flies (less than 24 h old), TRP levels are indistinguishable from control flies, but by 10 days the protein is barely detectable in immunoblots. In contrast, PLC-β, eye-PKC and other transduction protein levels remain constant. TRP channels are completely mislocalized in the *inaD*²¹⁵ mutant (Fig. 6), and are found randomly distributed throughout the plasma membrane (we assayed newly eclosed flies to prevent degradation of TRP). To examine the mislocalization of TRP in more detail, we performed immuno-electronmicroscopic staining using gold-conjugated secondary antibodies. These results (Fig. 6b) confirmed and extended the immunofluorescence observation that the TRP channel now localizes randomly to the plasma membrane. We found no evidence for mislocalization of any other phototransduction protein, including PLC-β and eye-PKC (Fig. 6 and data not shown). Also, contrary to previous reports³⁹, we never found TRP in the extracellular matrix, nor did we observe significant levels of cytoplasmic labelling.

To characterize the physiology of *inaD*²¹⁵ mutants we performed two sets of studies. Because immunoblot analysis revealed that levels of TRP protein decline with age in *inaD*²¹⁵ mutants, we predicted that *inaD*²¹⁵ cells should take on a TRP mutant phenotype over time. We found that *inaD*²¹⁵ mutants display an electroretinogram (ERG) phenotype that approaches that of *trp* mutants (Fig. 7a), and does so on a time course similar to that of the decay of TRP protein seen in immunoblots (data not shown). We also performed whole-cell voltage-clamp recordings of macroscopic currents and quantum

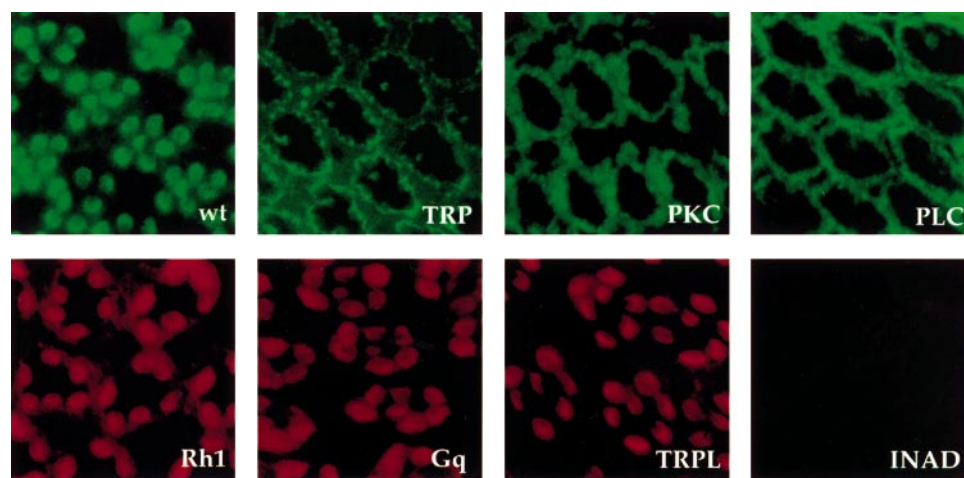


Figure 3 TRP, eye-PKC and PLC are mislocalized in *inaD*¹ null mutant photoreceptors. Immunofluorescent staining of cross-sections (1 μm thick) of wild-type (wt, top left) and *inaD*¹ mutant (all other panels) photoreceptors for different transduction proteins. Top, *inaD*¹ mutant photoreceptors show extensive

mislocalization of TRP, eye-PKC and PLC. Bottom, rhodopsin (Rh1), G_{qα} and TRP-like (TRPL) show normal rhabdomeric expression in *inaD*¹. Bottom right, the lack of Inad-immunoreactive material in the *inaD*¹ mutants.

bumps. The *inaD*²¹⁵ mutants were originally characterized as displaying slow deactivation kinetics in response to a flash of light³⁶ (Fig. 7b). This deactivation defect led others to postulate that InaD is involved in regulation of the phototransduction cascade, perhaps by modulating TRP function^{37–39}. To determine the basis for the slow deactivation component in *inaD*²¹⁵ photoreceptors, we characterized quantal responses. In wild-type photoreceptors, single photons give rise to unitary events known as quantum bumps^{41,42}. These quantum bumps are the result of the activation of a single rhodopsin molecule, and reflect the amplification of the entire signalling pathway, leading to the opening of the light-activated channels⁴³. Surprisingly, the quantum bumps from *inaD*²¹⁵ flies display normal termination kinetics (wild type, $t_{90\%} = 13.6 \pm 0.58$ ms; *inaD*²¹⁵, $t_{90\%} = 13.8 \pm 0.60$ ms; Fig. 7b, right), indicating that the macroscopic defect of *inaD*²¹⁵ mutants cannot be due to an underlying defect in deactivation. We reasoned that the apparent deactivation defect seen in the macroscopic response could be due to a bump activation defect. For instance, an increase in the latency of bump generation would be reflected as a broadly distributed macroscopic current⁴⁴. Indeed, we found that the mean latency times between stimulus and quantum-bump generation were significantly altered in *inaD*²¹⁵ mutants (wild type, 47.6 ± 1.3 ms; *inaD*²¹⁵, 67.0 ± 3.2 ms; Fig. 7c). Thus *inaD*²¹⁵ photoreceptors do not have a defect in termination³⁶, nor do they display problems with feedback regulation^{37,39}. Instead, the phenotype could be explained by understanding that the mislocalization of TRP channels leads to longer latencies and a corresponding macroscopic defect in deactivation kinetics.

To further define the physiological importance of the interaction

between InaD and its individual targets, we also analysed the interaction between InaD and PLC- β . Because *inaD*² has a mutation in the fifth PDZ domain (Fig. 1), and this domain is involved in the interaction with PLC- β (Fig. 2), we sought to determine whether *inaD*² photoreceptors display a specific redistribution of PLC- β *in vivo*. Indeed, PLC- β is randomly distributed in the cytoplasm of *inaD*² photoreceptor cells (Fig. 6), whereas other transduction proteins, such as eye-PKC, TRP, Rh1 and DG α , are unaffected. Consistent with its failure to be recruited into transduction complexes, PLC- β becomes unstable and decays over time (Fig. 5).

Given the loss of PLC- β from transduction complexes, we expected significant defects in phototransduction. ERG recordings from *inaD*² mutant photoreceptors are shown in Fig. 4. These cells exhibit major defects in response kinetics: latency, activation and deactivation are all significantly slower in the mutant cells. Because these recordings were performed in newly eclosed flies, a time at which there are near normal levels of PLC (Fig. 5), they demonstrate that it is not the presence of a transduction molecule, but rather its location, that promotes effective signalling. Taken together, our data indicate the existence of a highly organized signalling unit, a transducosome, show that it is possible to manipulate experimentally the composition of signalling complexes, and demonstrate that PDZ domains play an essential role in the assembly and function of transduction complexes *in vivo*.

Conclusions

Cells can respond to a vast array of signals, and do so by activating the appropriate intracellular signalling pathways. Many different receptors share common downstream targets, but it has not always

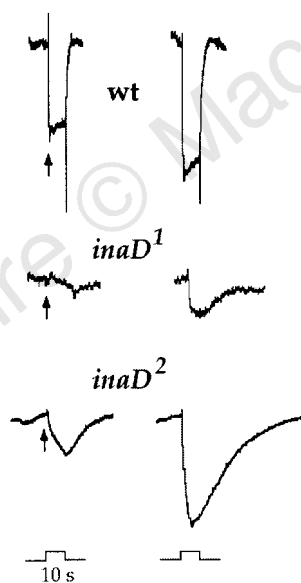


Figure 4 Photoresponses of *inaD*¹ and *inaD*². ERG recordings from wild type (wt), *inaD*¹ and *inaD*² mutant flies at <1 day after eclosion. The stimulus was a 10-s pulse of orange light (570 nm longpass filter). Right traces show responses to 10 times the amount of light in the left traces ($\log[I] = -2$ and $\log[I] = -1$, respectively). Note the severe defects in the light responses from the mutant cells. Arrows indicate the onset of the stimulus. Both mutants have an increase in loss of responsiveness as a function of age.

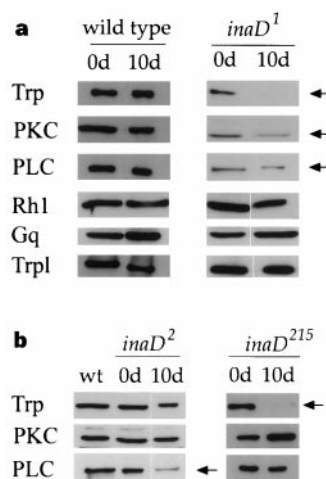


Figure 5 Transduction proteins that fail to associate with InaD become unstable. Immunoblot analysis of transduction proteins in wild type and *inaD* mutants. Protein levels at <24 h after eclosion (0 d) and after 10 days (10 d) are shown. **a**, Levels of all transduction proteins in wild-type flies remained constant with age, whereas TRP, eye-PKC and PLC declined drastically in *inaD*¹ mutants (see arrows). **b**, Only TRP declines in *inaD*²¹⁵ (PDZ3), and only PLC declines in *inaD*² (PDZ5) (see arrows). The equivalent of one fly head per lane was run for wild type and *inaD*²¹⁵, but two fly heads for *inaD*¹ and *inaD*².

been clear how different pathways maintain specificity. Several lines of evidence suggest that signalling events do not occur freely in the cytosol of the cell, but rather are organized as architecturally and spatially distinct ultra-microdomains of signalling. In this way, a cell can optimize and tune its responses to different pathways by controlling the recruitment of different signalling molecules into the different transduction complexes, thereby enhancing specificity and speed yet minimizing cross-talk. An example is the yeast mating response, in which the product of the *sterile 5* gene functions as a scaffold protein, coordinating the recruitment of several kinases within the same signalling pathway^{45–47}.

We have used phototransduction in *Drosophila* as a model to study the organization of G-protein-coupled transduction complexes *in vivo*. In this signalling pathway, photoreceptor neurons report activity with remarkable sensitivity and specificity. Furthermore, photoreceptors achieve excellent temporal resolution by ensuring that the transduction machinery is reset quickly after generating a response. Phototransduction in *Drosophila* is the fastest known G-protein-coupled cascade, taking just a few tens of milliseconds to go from light activation of rhodopsin to the generation of a receptor potential, and less than 100 ms to shut off after termination of the stimulus³⁰. InaD coordinates the recruitment of components involved in both activation (PLC- β and TRP) and deactivation (eye-PKC)³⁴. Our results suggest that an important strategy used by photoreceptors to attain high response speed is the assembly of signalling molecules into organized transducisomes. Therefore response time would not be limited by the diffusion of different signalling components within a microvillus.

A photoreceptor neuron attains high sensitivity by having an

exceptionally large number of receptor molecules in its surface ($\sim 100 \times 10^6$ rhodopsins per cell)^{48,49}. To couple this large number of receptor molecules to the downstream transduction complexes rapidly and efficiently would require a diffusible coupling molecule, in this case a G protein. In this model, each G protein would need to sample only a small number of receptors in the membrane (an ultra-microdomain of signalling) and report their activity to the downstream transduction complexes. Our finding that neither rhodopsin or G α are included in the InaD complexes supports this model. What do these complexes tell us about unitary (single-photon) responses? A quantum bump represents the coordinated activation of a few hundred light-activated ion channels in response to the activation of a single rhodopsin molecule³⁰. The organization of InaD complexes into a supramolecular complex, either through PDZ–PDZ domain interactions or PDZ–cytoskeletal interactions within a microvillus, could represent the structural basis of a quantum bump, ensuring both reliability and coordinated signalling. Our observation that PLC is positioned in close proximity to the light-activated channels through InaD provides an intriguing possibility for a direct gating mechanism. Also, the finding that the TRPL channels remain properly localized in *inaD* mutants further supports the postulate that TRP and TRPL are not likely to form or function as heteromultimers *in vivo*³³.

The structural basis of the interaction between PDZ domains and their targets is becoming clear. For instance, the X-ray crystallographic structure of the third PDZ domain from the synaptic protein PSD-95, alone or in complex with its peptide ligand, has recently been determined^{5,27,28}. In addition, binding studies with oriented peptide libraries showed that different PDZ domains

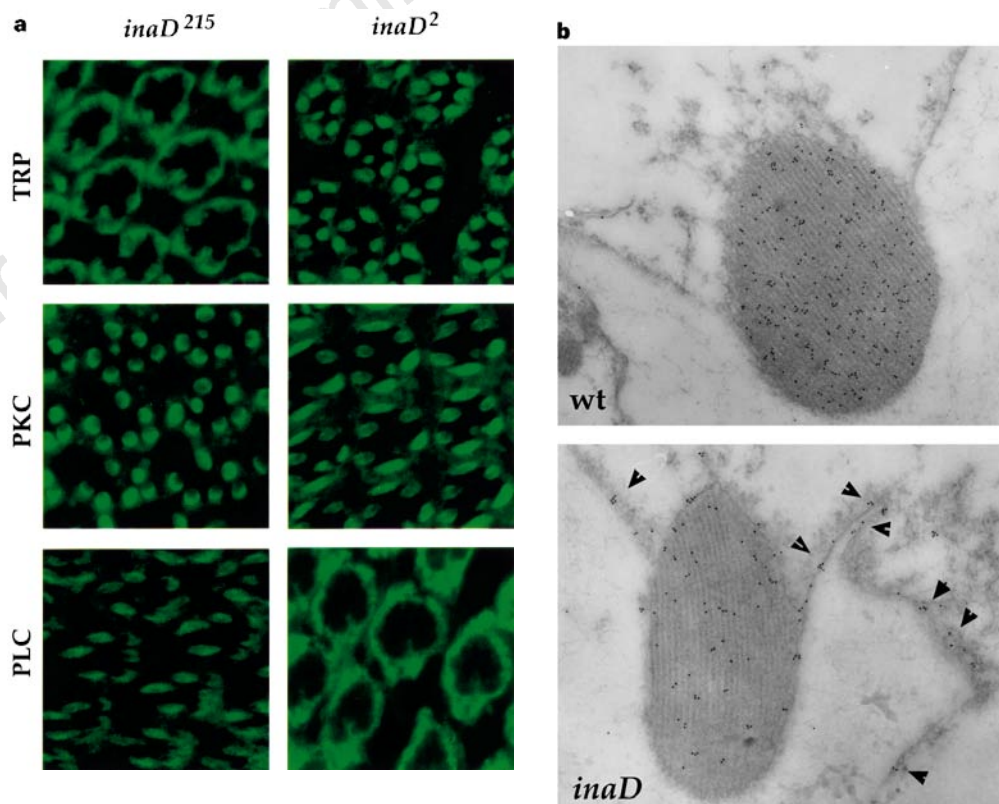


Figure 6 *inaD*²¹⁵ and *inaD*² mutants show specific mislocalization of TRP and PLC. **a**, Immunofluorescent staining for TRP, eye-PKC and PLC (as indicated) in cross-section (1 μ m thick) of *inaD*²¹⁵ (left) and *inaD*² (right) mutant photoreceptors. TRP is mislocalized in *inaD*²¹⁵ mutants, whereas PLC is mislocalized in *inaD*² mutants. All other transduction proteins show normal rhabdomeric localization in either genetic background. **b**, Electronmicroscopic immunogold localization of

TRP in wild-type (wt) and *inaD*²¹⁵ rhabdomeres^{31,33}. In wild-type photoreceptors, TRP is present at significantly higher levels and exclusively in the microvillar membranes of the rhabdomeres; in *inaD*²¹⁵, TRP levels are significantly reduced in the rhabdomeres (newly eclosed flies), and is distributed prominently throughout the plasma membrane (arrows).

display preferences for distinct targets⁴⁰. These *in vitro* studies suggested that, although all PDZ domains may share structural elements, different PDZ domains should show specificity for different target proteins. We have now shown this to be the case *in vivo*. The finding that InaD is composed primarily of PDZ domains strengthens the importance of this protein motif in the organization of signalling pathways. Recently, GRIP (glutamate receptor interacting protein), a new protein composed solely of seven PDZ domains, has been identified in mammalian cells⁵⁰. It has not yet been determined whether this molecule also functions as a

signalling scaffold, organizing specific transduction complexes at the synapse. Current models of PDZ–target interaction involve a binding site composed of S/TXV residues at the carboxy-terminal end of the target protein. All three InaD targets lack such a motif (position –4 from the C terminus in TRP, positions –122 and –507 in eye-PKC, and several sites PLC- β , the closest at residue –26), which argues against an exclusive model requiring that PDZ domains bind the absolute C-terminal end of their target proteins^{5,27,29,40}. We also showed that InaD functions as a modular protein, and demonstrated that eliminating one target does not prevent InaD from interacting with the others. The identification of all of the InaD targets and the corresponding PDZ sites should provide an experimentally tractable system in which to define the structural basis of PDZ–target interaction *in vivo*. It may even be possible to custom-design transduction complexes by manipulating the number, orientation and distribution of PDZ domains in InaD. The availability of a null *inaD* mutant background now makes these studies possible. □

Methods

Mutant screen and western blots. Males of *Drosophila* *cn bw* genotype were aged for 5 days, treated with EMS, and crossed to flies carrying the dominant temperature-sensitive *DTS91* allele. Single F₁ males were collected and crossed in single vials to CyO/*DTS91* virgin females. The vials were then shifted to 29 °C for 72 h to eliminate any eggs or larvae carrying the *DTS* allele. The parents were then removed and the vials were incubated at 29 °C for an additional 48 h before being returned to 25 °C. The progeny from this cross were transferred to fresh food, and their homozygous white-eyed offspring (*cn bw*) were subjected to a protein immunoblot screen for the loss of the InaD antigen³³.

Antibodies. To generate antibodies specific to InaD, we generated a T7 fusion protein consisting of the last 300 residues of the protein. All antibodies were checked for specificity and affinity using wild-type, mutant and transgenic controls. For immunostaining, the InaD antibody was diluted 1:500 in phosphate buffered saline (PBS), 1% BSA, 0.1% saponin (PBS-S); the TRP antibody was first preabsorbed with a homogenate of *trp* mutant heads to reduce background staining and used at a final dilution of 1:100. Rhodopsin (1:300), eye-PKC (1:50), PLC (1:1000), TRPL (1:100) and DG α (1:200) were detected using polyclonal antibodies^{33,34,44}.

Immunoprecipitation. Frozen heads (500–1,000) were homogenized in 2–3 ml of buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA and protease inhibitors) using a glass–glass homogenizer. The homogenate was centrifuged at 4,000g for 1 min to remove chitinous material. Membranes were isolated by centrifugation at 100,000g for 30 min at 4 °C, and resuspended in 0.8–1.0 ml of buffer A to determine protein concentration. Samples were recentrifuged, resuspended in buffer B (150 mM NaCl, 1% triton, 50 mM Tris-HCl, pH 8.0, and protease inhibitors), mixed (100 μ g of protein) with anti-InaD antibodies, and incubated for 1 h at 4 °C. At this time, 30 μ l of protein A-agarose beads (Pierce) were added and incubated for 2 h. Samples were washed in buffer B, resuspended in SDS buffer and fractionated by SDS–PAGE. The entire immunoprecipitate was loaded on the gels. Studies using GST–InaD protein fusions used similar incubation conditions but also contained affinity-purified GST fusion proteins. GST fusions containing individual PDZ domains (PDZ1 to PDZ5) were constructed according to the boundaries shown in Fig. 1. All GST fusion proteins were overproduced and purified by affinity chromatography on glutathione-agarose beads^{19,23,50}.

Electrophysiological recordings. Photoreceptors were isolated from adult flies (<6 h after eclosion) and whole-cell, patch-clamp recordings were performed³³. Photoreceptors were stimulated by a 75-W Xenon source connected to the epifluorescence port of an inverted Fluovert FS (Leitz) microscope; light was bandpass-filtered ($\lambda = 580 \pm 10$ nm) and focused onto the photoreceptor cells with a 0.5 numerical aperture, 40 $\times$ objective. Signals were recorded with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA), and data were analysed using PCLAMP6.02 (Axon) and ORIGIN (Microcal) software. The membrane of the photoreceptors was voltage-clamped at a holding potential of –40 mV. Traces were low-pass filtered at 2 kHz (Bessel filter) and digitized at 2 kHz, unless stated otherwise. Measured series resistance, 16 M Ω on average, was 80% compensated. The bath solution

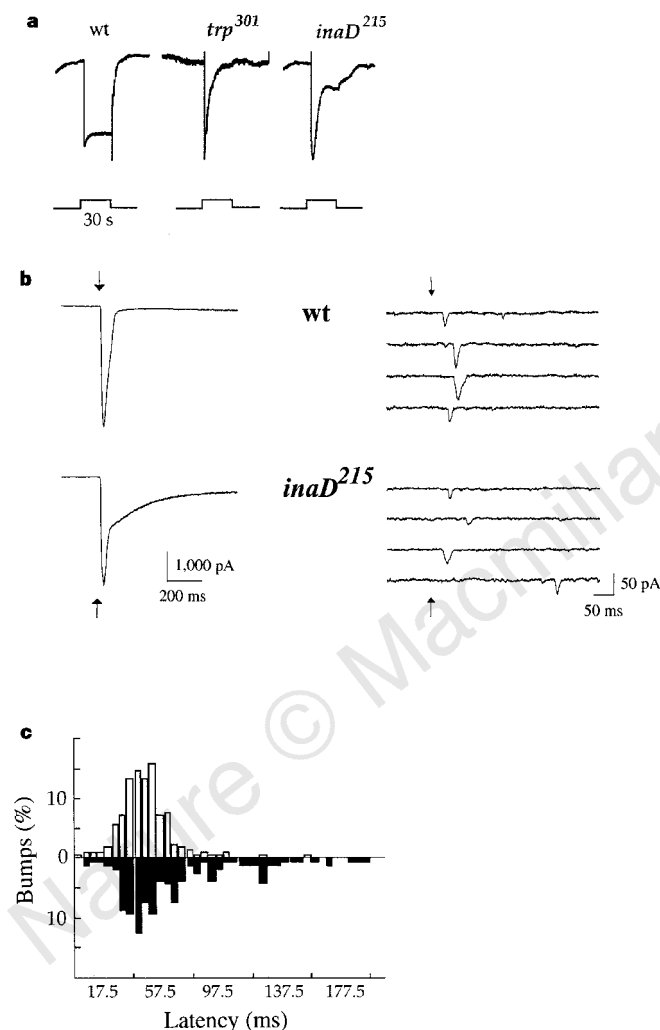


Figure 7 Photoreponses of *inaD*²¹⁵. **a**, ERG recordings from wild-type (*wt*), *trp*³⁰¹ and *inaD*²¹⁵ mutant eyes at 20 days after eclosion. Light stimulus was a 30-s pulse of orange light (570 nm longpass filter). Note the transient response of *trp* (transient receptor potential) mutants and older *inaD*²¹⁵ flies. **b**, Whole-cell recordings (left) and quantum bumps (right) from wild-type and *inaD*²¹⁵ mutant photoreceptors. For macroscopic responses, cells were stimulated (arrow) with a 10-ms flash of 580 nm light of $\log[I] = 1$. The deactivation time course of *inaD*²¹⁵ is well fitted by the sum of two exponentials (time constants of 14.7 ± 2.5 and 143.1 ± 12.1 ms; $N = 7$), whereas the time course of decay of wild-type responses is fitted by a single exponential with a time constant of 14.5 ± 2.2 ms ($N = 6$). For quantum bumps⁴⁴, cells were stimulated with a 10-ms flash of 580 nm light of $\log[I] = -6.5$ (arrow). This stimulus produced a probability of not seeing a bump of 0.40 in wild type and 0.65 in *inaD*²¹⁵. Note the normal termination kinetics of *inaD*²¹⁵ bumps. **c**, *inaD*²¹⁵ quantum bumps have defective latency. Latency to first bump from wild type (open bars) and *inaD*²¹⁵ mutant (solid bars) were 47.6 ± 1.3 ms ($N = 210$ bumps from 7 cells), and 67.0 ± 3.1 ms ($N = 160$ bumps from 7 cells), respectively.

contained (in mM): 124 NaCl, 4 KCl, 10 HEPES, 5 proline, 25 sucrose, 1.5 CaCl₂, 1 MgCl₂, pH 7.15. Pipette solution contained 95 K-gluconate, 40 KCl, 10 HEPES, 2 MgCl₂, 2 EGTA, pH 7.15. For quantum-bump analysis, photoreceptors were clamped at -70 mV, and stimulated with a dim light flash to generate quantum bumps around 50% of the time. Signals were lowpass-filtered at 1 kHz and digitized at 2 kHz.

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A high deuterium abundance at redshift $z = 0.7$

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Of the light elements, the primordial abundance of deuterium relative to hydrogen, $(D/H)_p$, provides the most sensitive diagnostic¹ for the cosmological mass density parameter, Ω_B . Recent high-redshift D/H measurements are highly discrepant²⁻⁶, although this may reflect observational uncertainties^{7,8}. The larger primordial D/H values imply a low Ω_B (requiring the Universe to be dominated by non-baryonic matter), and cause problems for galactic chemical evolution models, which have difficulty in reproducing the steep decline in D/H to the present-day values. Conversely, the lower D/H values measured at high redshift imply an Ω_B greater than that derived from ⁷Li and ⁴He abundance measurements, and may require a deuterium-abundance evolution that is too low to easily explain. Here we report the first measurement of D/H at intermediate redshift ($z = 0.7010$), in a gas cloud selected to minimize observational uncertainties. Our analysis yields a value of D/H $((2.0 \pm 0.5) \times 10^{-4})$ which is at the upper end of the range of values measured at high redshifts. This finding, together with other independent observations, suggests that there may be inhomogeneity in $(D/H)_p$ of at least a factor of ten.

Measurements of the abundance of deuterium can be achieved using absorption-line spectroscopy of gas clouds intersecting the sight-lines to distant, background quasars by measuring strengths of the absorption lines of the H I and D I Lyman series. The D I transitions occur at an isotopic shift of -81 km s^{-1} with respect to the H I series. Such a measurement is observationally difficult for a number of reasons^{9,10}: (1) ill-placed H I Lyman- α -forest absorption lines can masquerade as deuterium and (2) most quasar absorption systems show complex internal velocity structure, which may render parameter estimation for individual components of interest unreliable or impossible. To overcome the first difficulty, we searched for suitable absorbers at intermediate redshifts (that is, at $z < 1$), where the number density Lyman- α -forest systems is far lower than at high redshifts. To overcome the second difficulty, we searched for suitable absorbers with apparently simple velocity structure. We found one object which appears optimal for a D/H analysis; only one absorbing component is revealed by the data and the velocity dispersion in that component is small enough to easily detect and accurately measure the deuterium abundance.

The absorber at $z = 0.7010$ towards the quasar 1718 + 4807 (of emission redshift $z_{\text{em}} = 1.084$ and apparent magnitude $m_V = 15.3$) was selected on the basis of a remarkably abrupt partial Lyman-limit discontinuity in the spectrum¹¹ measured by the International Ultraviolet Explorer (IUE) satellite. The extreme sharpness of the Lyman break clearly indicates simple velocity structure and low velocity-dispersion parameter. This simple velocity structure contrasts with the complex nature of the higher-redshift systems reported so far, both those which give low D/H measurements^{2,3,7} and those which give high values⁴⁻⁶, so the measured column

densities (based on Lyman- α and the Lyman limit alone) are likely to be more reliable than in those cases. Hubble Space Telescope (HST) spectra were obtained of the spectral region covering the Lyman- α and Si III transitions using the Goddard High Resolution Spectrograph and the G270M grating. All spectra were extracted, binned to linear wavelength scales and corrected to vacuum, heliocentric wavelengths using standard procedures. Absorption-line parameter estimates were derived using VPFIT¹², a computer program based on Gauss-Newton unconstrained optimization. Parameter errors were derived from the diagonal terms of the parameter covariance matrix computed at the best fit. The data and model fits are shown in Fig. 1.

The value of D/H that we derive from the combined data is $(2.0 \pm 0.5) \times 10^{-4}$ which is approximately an order of magnitude larger than recent ground-based measurements of high-redshift absorbers^{2,3,7} (although the reliability of these measurements is at

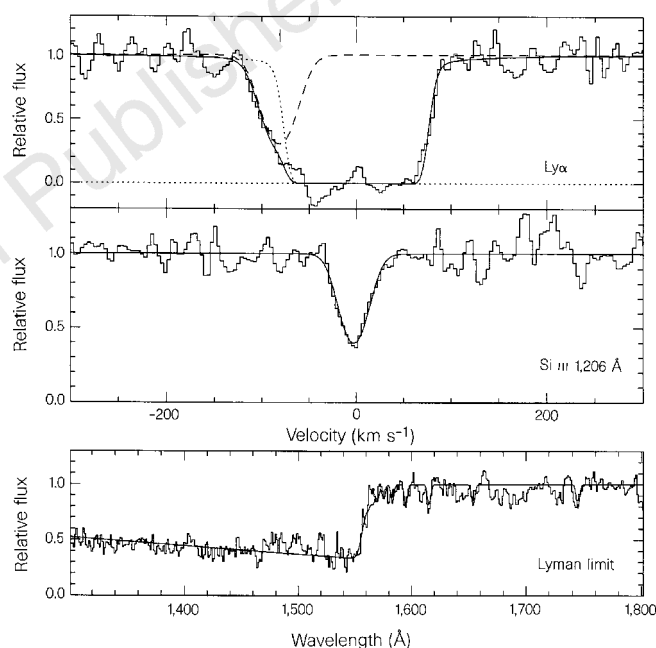


Figure 1 Data and model fits for Q1718 + 4807. Top panel, HST H I and D I profile, and model profiles for H I (dotted line), D I (dashed line) and H I + D I (solid line). Vertical line segments indicate wavelength centroids of H I and D I. Middle panel, HST Si III 1,206.51 Å profile and fit. The HST spectrum was observed on 3 March 1995 (total integration time, 282 min). The spectral resolution is 20,000. Bottom panel, IUE spectrum and fit. The pixel size is 1.18 Å and the adopted spectral resolution is 2.5 pixels. To minimize the number of free parameters, we adopted a single redshift for H I, D I and Si III lines and the Lyman limit. The parameter constraints arise as follows. The IUE spectrum provides an accurate determination of the H I column density ($\log N(\text{H I}) = 17.24 \pm 0.01 \text{ cm}^{-2}$). The Si III 1,206 absorption line supports the single-component velocity structure implied by the IUE Lyman limit and determines the cloud redshift precisely ($z_{\text{abs}} = 0.701024 \pm 0.000007$). The Ly α absorption is clearly asymmetric, showing additional absorption in the blue wing at the position corresponding to D I. As Si III accurately constrains the D I position and because the Doppler parameter (which is equal to $\sqrt{2}$ times the r.m.s. velocity dispersion) $b(\text{D I})$ is constrained by the overall fit, only one free parameter, $N(\text{D I})$, is required to fit the excess absorption seen at the D position. The D I column density is thus accurately determined and is $\log N(\text{D I}) = 13.57 \pm 0.06 \text{ cm}^{-2}$. The Doppler parameters are dominated by non-thermal broadening with an inferred temperature of $1.9 \times 10^4 \text{ K}$ and $b(\text{H I}) = 25.5 \text{ km s}^{-1}$, $b(\text{D I}) = 22.2 \text{ km s}^{-1}$ and $b(\text{Si III}) = 18.7 \text{ km s}^{-1}$, with the same error on each of $\sigma = 0.5 \text{ km s}^{-1}$. From the above, we derive $D/H = (2.0 \pm 0.5) \times 10^{-4}$.

present a matter of debate⁸) and an order of magnitude larger than a recent intermediate-redshift measurement¹³ at $z = 0.5$. It is of course possible that our high value is caused by a weak H I line which just happens to fall very close to the D I wavelength. Unfortunately, a posteriori statistics do not provide a reliable means of assessing the probability of this because the absorption cloud we have studied was specifically selected as having uniquely simple velocity structure, a property which itself implies a low probability of an H I interloper successfully mimicking D I. We note, however, that the probability of a randomly placed interloper mimicking deuterium is extremely small; using the observed number density of absorbers in the spectrum and adopting a generous 4σ tolerance on the line position, it is $<1\%$.

To examine this possibility further, we re-fitted the data replacing D I with H I (so that the redshift of the putative interloper was a free parameter). The best fit resulted in the new H I line falling at $-86 \pm 5 \text{ km s}^{-1}$ from the strong component, implying that the detected absorption feature is deuterium and not an interloper. Furthermore, the Doppler parameter of this putative H I interloper ($b = 21 \pm 4 \text{ km s}^{-1}$) lies between that of the strong H I component and that of the Si III value, which is again consistent with the expectation for D I. Taken together, these points indicate that the most reasonable and likely interpretation of the data is that we have detected D I.

A further possibility is that some other transition from an absorbing cloud at some other redshift just happens to coincide with the D I wavelength at the absorption redshift $z_{\text{abs}} = 0.701024$. The closest candidate is interstellar Cr II 2,066.16 Å. The D I feature falls at 2,067.31 Å. This corresponds to a shift of $\sim 170 \text{ km s}^{-1}$, so Cr II 2,066.16 Å is not a serious contender for D I contamination. Furthermore, Cr II 2,056 Å is not observed and yet has an oscillator strength twice that of Cr II 2,066.16 Å. We conclude that no Cr II contamination occurs.

We cannot reliably estimate the heavy-element abundances of the $z_{\text{abs}} = 0.701024$ system, as the only species in the observed range is Si III (of column density $\log N(\text{Si III}) = 12.81 \pm 0.04 \text{ cm}^{-2}$). An upper limit can be derived, but this only constrains the abundances to be less than solar; they could easily be very much lower. We note that it would be surprising if the $z = 0.701024$ system turned out to have already undergone substantial chemical evolution and yet exhibit such a high D/H ratio. Future observations of any unsaturated absorption lines in this system (Mg II or Fe II, for example) should yield reliable constraints on heavy-element abundance. Astration (deuterium destruction in stars) means that any particular D/H detection provides a lower limit to the primordial value. Therefore, because our measured D/H is high, the lack of information about the heavy-element abundances does not alter the interpretation of the data described below.

In standard models of galactic chemical evolution, the mass fraction of deuterium decreases steadily with time, by a factor $\sim 2\text{--}3$ over 10 Gyr, so that when plotted versus metallicity, it appears constant until a metallicity of one-tenth solar and drops abruptly thereafter¹⁴. The two extremes of the recent high-redshift D/H observations imply radically different consequences for the baryonic mass density parameter Ω_B , and its cosmological significance^{15,16}. A high $(\text{D}/\text{H})_p$ would be in excellent agreement with standard homogeneous Big-Bang nucleosynthesis (BBN) models and the observed 'primordial' abundances of ^4He and ^7Li , but observations of D/H in the interstellar medium then require destruction of deuterium by a factor ~ 10 up to the present epoch. Whether this much astration can be easily explained by chemical evolution models is unclear, although some recent models may succeed¹⁷.

A D/H ratio of 2.0×10^{-4} , as obtained here towards Q1718+4807, corresponds to a mass fraction of ^4He (Y) of 0.233 ± 0.002 , $^7\text{Li}/\text{H} \approx (1.8^{+0.7}_{-0.6}) \times 10^{-10}$ and $\Omega_B \approx 0.006 \pm 0.003 h^{-2}$, where h denotes the value of the Hubble constant in

units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and the errors only include 1σ errors on the nuclear cross-sections of BBN (values were derived using fitting formulae¹⁸). These figures agree precisely with the measured abundances, $Y \approx 0.233 \pm 0.005$ and $^7\text{Li}/\text{H} \approx (1.8^{+0.5}_{-0.3}) \times 10^{-10}$. The above baryonic mass density parameter may be compared with the measured amount of visible matter in galaxies, $\Omega_{\text{vis}} \approx (0.002\text{--}0.005) h^{-1}$. This shows that a high primordial deuterium abundance leaves little room for baryonic dark matter and supports the view that the missing mass inferred from dynamical studies must be non-baryonic. On the other hand, a low $(\text{D}/\text{H})_p$ implies BBN values of ^4He and $^7\text{Li}/\text{H}$ which differ from the measured values by amounts corresponding to 2σ observational limits (although it avoids the difficulty in explaining such rapid deuterium destruction, a value which is too low may still be problematic if the implied astration is too small compared to predictions).

Several explanations have been put forward which could explain discrepant values of the D/H ratio. Post-BBN chemical evolution processes might result in strong deuterium depletion in some gas clouds, giving rise to low observed abundances. By redshifts $z \approx 3\text{--}4$, stars down to about two solar masses ($\sim 2 M_\odot$) have had time to eject deuterium-poor gas. But the observational upper limits on C/H and Si/H in clouds at high redshift where $\text{D}/\text{H} \approx 2.5 \times 10^{-5}$ imply metal abundances $\leq 10^{-2}$ solar, constraining the fraction of gas which has been cycled through stars to be $\leq 5\%$. Such models seem to offer reasonable compatibility with observed light-element abundances only by admitting unreasonable stellar populations, such as a primordial population of supermassive stars of mass $M \geq 1,000 M_\odot$, or an initial mass function strongly peaked around $M \approx 6 M_\odot$.

Alternatively, primordial isocurvature baryon fluctuations¹⁹ could account for a variation of the $(\text{D}/\text{H})_p$ ratio by a factor ~ 10 , but only on scales corresponding to a Jeans mass of $\sim (10^5\text{--}10^6) M_\odot$. For a specific class of these models, there are opposing views as to whether or not the observed isotropy of the cosmic microwave background rules out substantial intrinsic D/H fluctuations^{19–22}. We note, however, that homogeneous, critical-density models with a low baryonic component, $\Omega_B < 0.01 h^{-2}$, seem to predict degree-scale microwave background fluctuations which are significantly less than those actually observed²³. Also, assuming no segregation between baryons and dark matter²⁴, X-ray observations of galaxy clusters support this point²³ because, for critical-density models, they independently suggest baryonic density parameters of $\Omega_B > 0.02 h^{-3/2}$. Both these types of observation therefore seem to be in conflict with a baryonic density parameter for a homogeneous Universe as low as that derived from our results for D/H in the $z_{\text{abs}} = 0.701024$ gas cloud reported here. Our results therefore indicate that either the Universe does not have a critical total density, or, if it does, Big-Bang nucleosynthesis must have occurred inhomogeneously. □

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Chaotic dynamics of falling disks

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The study of the motion of flat bodies falling in a viscous medium dates back at least to Newton¹ and Maxwell², and is relevant to problems in meteorology³, sedimentology⁴, aerospace engineering¹ and chemical engineering^{5–8}. More recent theoretical studies^{9–12} have emphasized the role played by deterministic chaos, although many experimental studies^{1,5–8,13,14} were performed before the development of such ideas. Here we report experimental observations of the dynamics of disks falling in water/glycerol mixtures. We find four distinct types of motion, which are mapped out in a ‘phase diagram’. The apparently complex behaviour can be reduced to a series of one-dimensional maps, which display a discontinuity at the crossover from periodic to chaotic motion. This discontinuity leads to an unusual intermittency transition¹⁵, not previously observed experimentally, between the two behaviours.

Steel and lead disks with diameters $d = 5.1$ – 18.0 mm and thicknesses $t = 0.076$ – 1.63 mm were dropped in water and water/glycerol mixtures from heights ranging from ~ 0.3 to 1 m; some paper disks were also dropped in air, as discussed later. This combination of disk sizes and densities, and fluid viscosities, allowed us to explore a very large region of parameter space. Several different types of motion were observed (Fig. 1).

We now consider the system parameters that determine the type of motion observed. There are five material parameters: the disk diameter d , thickness t and density ρ , as well as the fluid density ρ_f and kinematic viscosity ν . From these five, three independent dimensionless ratios¹³ may be formed. The first is the dimensionless moment of inertia $I^* = I_{\text{disk}}/\rho_f d^5 = \pi \rho t/64 \rho_f d$. For small I^* , we

expect the effects of the fluid to be important; at large I^* the moment of inertia of the disk dominates. A second dimensionless quality is the Reynolds number $Re = Ud/\nu$, where U is the mean vertical disk velocity. Finally, for the disks we consider here, the quantity t/d is small, and we expect therefore that this ratio plays no role in the disks’ dynamics.

To investigate the dependence of the disks’ behaviour on two dimensionless parameters I^* and Re , we have dropped a large number of disks and visually characterized their motion as ‘steady falling’, ‘periodic oscillating’, ‘chaotic’ or ‘tumbling’. We may then plot a ‘phase’ diagram, indicating the behaviour of a disk as a function of I^* and Re (Fig. 2). Also plotted on this diagram are data from refs 13 and 14, which agree quite well with ours. We note that there is a well defined boundary between each regime. This implies that the two dimensionless quantities I^* and Re do in fact characterize a disk/liquid combination.

Mapping out a phase diagram by direct observation allows a compact overview of the disks’ behaviour. However, it does not give us any physical insight as to why the disks fall as they do. To explore this question more fully, we have performed direct video imaging of the trajectories of disks as they fall. From such images, it is possible to obtain the two cartesian coordinates of a disk’s centre in the vertical plane perpendicular to the direction of observation, as well as the direction in space of the symmetry axis of the disk. The cartesian coordinate parallel to the direction of observation, and the angle of rotation around the symmetry axis, could not be recorded. A simple analysis of this motion would seem difficult, then, as the disk has six degrees of freedom, of which we record four.

We have found it possible to describe this complex behaviour by a simple one-dimensional map. The single variable required is the angle θ between the disk’s normal and the vertical. To understand this, imagine dropping a disk from rest in a fluid, starting at an angle θ_0 . As seen in Fig. 1a, the disk will glide downwards and to the side, level off, and then begin to tilt in the other direction. Just when it has reached the extremum of its motion (indicated by the arrow in Fig. 1a) the disk is at a well-defined final angle θ_f . As the motion is deterministic, this final angle θ_f must be a function f of its initial conditions—the initial angle equalling θ_0 and the other coordinates and velocities being zero. Thus, it must be true that $\theta_f = f(\theta_0)$.

We may now ask the question as to whether this final angle θ_f may serve as the initial angle θ_i for the next oscillation, with the next θ_f determined from the same mapping f as above. This will be possible to the extent to which this angle alone completely specifies the initial conditions, even for a disk which has fallen quite far. How might this be so? At an extremum (Fig. 1) the angular velocities are essentially zero. So, too, are the horizontal translation velocities. The vertical velocity, although not zero, very rapidly reaches a terminal value which is about the same for all subsequent extrema. Certainly the disk’s centre-of-mass coordinates are irrelevant if we consider the fluid to be infinite. We also expect from symmetry considerations that effects of rotations around the disk’s axis will be small. Thus, the only relevant disk coordinate is the angle θ between the disk’s plane and the horizontal.

The effect of the infinite degrees of freedom of the fluid is, of course, much more difficult to consider. A detailed study of the fluid dynamics as the disk approaches a given θ_f would undoubtedly reveal vortex shedding, turbulence and other complex motion. Without knowing the details of the motion of the fluid, however, we can imagine that for a repetitive motion like the periodic oscillation of a disk, the motion of the fluid in the vicinity of the disk also repeats itself. Thus, we can consider the fluid motion to be included in our description of the state of the system, which we describe by the single variable θ_f . This state is still determined by the previous θ_i , even though it is determined by extremely complicated dynamics. We can thus think of $f(\theta_i)$ as a single-valued function, and can map it out empirically.

This idea of including the fluid dynamics in some variable which

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ostensibly describes the disk is not a novel one. For the related case of a body moving in an ideal fluid, Kirchhoff showed (see, for example, ref. 9) that the equations of motion for such a body can be written so that the infinite degrees of freedom of the fluid are eliminated; they still influence the body dynamics by way of the added mass tensor, whose elements depend only on the shape of the body.

In Fig. 3, we show eight experimental iteration maps of θ_f versus θ_b for several disk/liquid combinations. Maps shown in Fig. 3a–d correspond to those points in the phase diagram of Fig. 2 labelled a–d. We see that the latter form a vertical slice in phase space which cuts from the periodic-oscillation region of low I^* through the boundary with the chaotic region of higher I^* . The progression of the

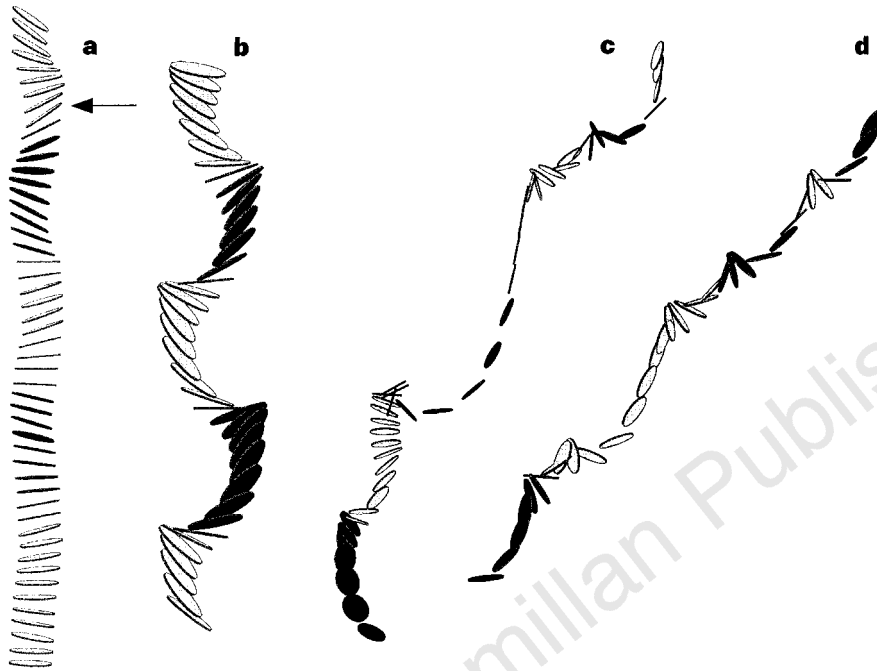


Figure 1 Trajectories of falling disks, obtained by imaging from the side using a video camera; the digitized images were measured, and computer-drawn images are shown. One side of each disk is drawn white, the other black. **a**, Steady-falling regime observed at lower Reynolds' numbers (Re): a disk, dropped with any initial orientation, quickly settles down to a steady fall with horizontal orientation. The arrow represents an extremum of motion. **b**, Periodic-oscillating motion observed at higher Re and low dimensionless moment of inertia I^* . Such disks oscillate with a well-defined period, again settling into this pattern after initial transients have died down. **c**, Chaotic motion found when both Re and I^* are moderately large. Typically, a disk in this regime will begin to oscillate with larger and larger amplitude until its angle is so high that it actually flips over. It then tumbles several times and then suddenly jumps back to oscillating behaviour. The number of tumbles and time between tumbling behaviours appear to the eye to be random. The disk may also suddenly change its overall direction of motion. **d**, Tumbling motion found at very large I^* . Here, the disk turns continuously end-over-end while drifting in one direction.

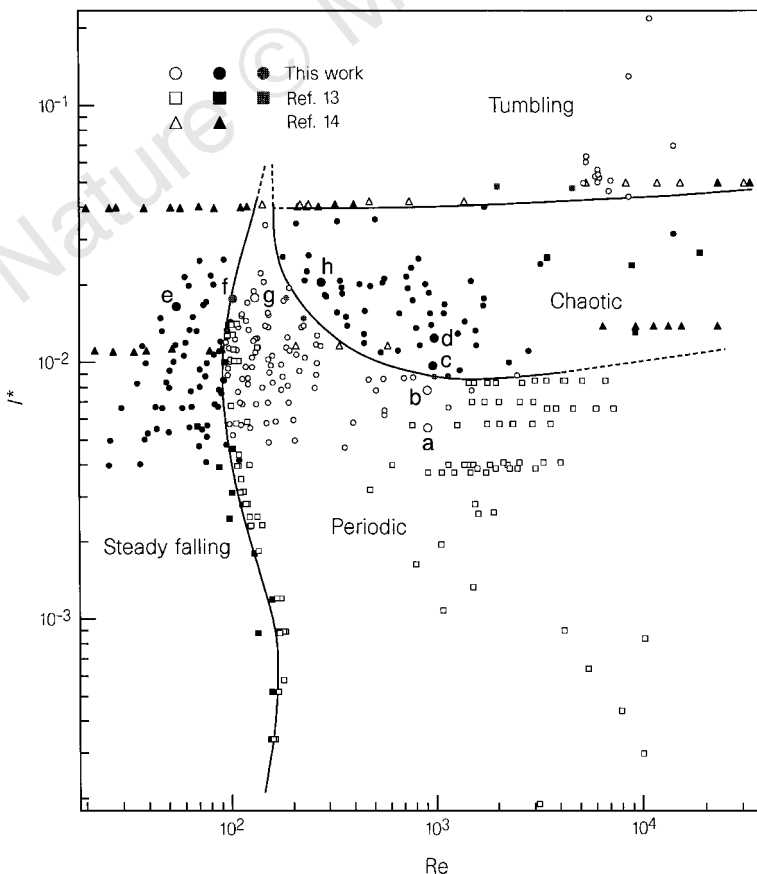


Figure 2 Phase diagram showing the dynamical behaviour of the disks as a function of the two parameters I^* (dimensionless moment of inertia) and Re (Reynold's number). Filled symbols represent disks with steady falling or chaotic behaviour, and open symbols periodic or tumbling behaviour. Grey symbols represent cases judged to lie on the border between two regimes. The lettered points, enlarged for clarity, correspond to iteration maps shown in Fig. 3a–h.

maps indicates how this change in dynamical behaviour proceeds. In Fig. 3a we see that, near an angle of about $\theta_0 \approx 60^\circ$, the map cuts across the diagonal line $\theta_f = \theta_i$ with a slope less than one. θ_0 thus represents a stable fixed point, and the motion will always settle down to periodic oscillations with an extremum angle of about 60° . As we increase I^* , we come to point b in Fig. 2, which is near the border between periodic and chaotic behaviour. In the iteration map of Fig. 3b, we see that there appears to be a stable fixed point, although it is very near to 90° . Thus, at the end of each oscillation, the disk is nearly vertical, and is quite near to actually flipping over. Increasing I^* further, we come to point c in Fig. 2, which is now clearly in the chaotic region. The map (Fig. 3c) no longer crosses the diagonal, so there are no fixed points. Instead, a disk dropped with any initial angle very rapidly increases its successive angles until $\theta \geq 90^\circ$. We see from the map, however, that there is no final angle defined for initial angles greater than about 80° ; for initial angles greater than this the disk will actually flip over. We find that it then tumbles several times; this number is unpredictable. The disk is then suddenly reinjected into the map at some low angle θ , and the process begins again. This progression from oscillations near a fixed point to chaotic behaviour has been termed intermittency¹⁵. Finally, for larger I^* (Fig. 3d), the map is very far from the diagonal line, and the motion is strongly chaotic. Oscillation angles rapidly build towards 90° , and the disk alternates rapidly between tumbling and oscillating. We have schematically indicated this complicated tumbling behaviour by the rapidly fluctuating curve for $\theta \geq 67^\circ$ in Fig.

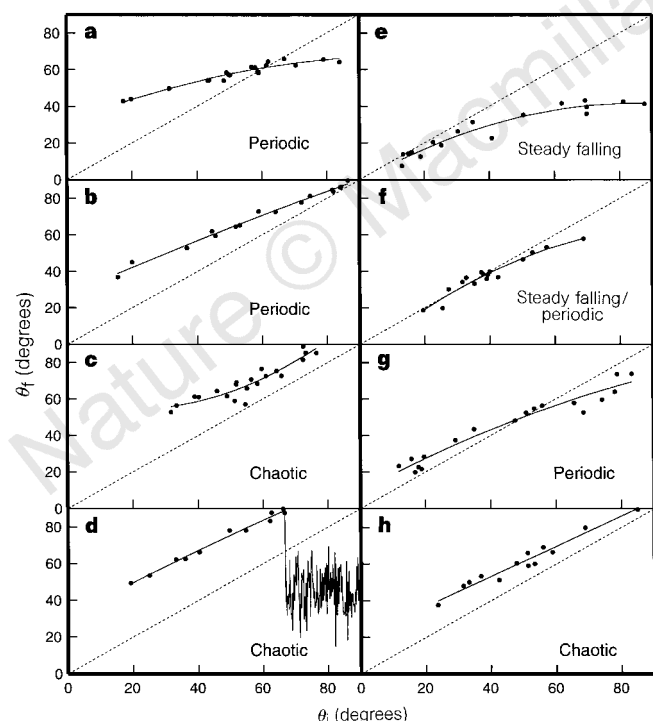


Figure 3 Iteration maps of falling disks, constructed as follows. A disk was dropped from rest with some initial angle θ_0 . From the resulting digitized motion, a sequence of angles at the extrema of motion was measured. Each angle serves as an initial angle θ_i for the next final angle θ_f . By starting out at several different values of θ_0 , a rather wide range of angles could be mapped out. To avoid transients, we did not include the initial angle from which the disk was dropped at rest in constructing these maps, making small initial angles sometimes difficult to achieve—the angle after even the first iteration was often already large. Sequence **a–d** represents a roughly vertical slice through I^* -Re space, and **e–h** a horizontal slice. The curves are guides to the eye. The diagonal dashed lines of slope one meet the curves at the fixed points where $\theta_i = \theta_f$.

3d. A similar route to chaos is observed when taking horizontal slices through the phase diagram, as in e–h in Fig. 2, corresponding to maps shown in Fig. 3e–h.

Pomeau and Manneville classify three types of intermittency depending on the way fixed points become unstable¹⁵. Most experimental systems exhibiting intermittency appear to be of one of these three types. This classification, however, does not cover maps that are not differentiable around fixed points¹⁶. Here, a fixed point can lose its stability by colliding with a discontinuity in the map. After passing the point of discontinuity, the iteration is reinjected 'stochastically' into the laminar region. We observe this 'type V' intermittency in our experiments.

As we enter the chaotic regime, a discontinuity indeed develops in the map (Fig. 3c, d) at that value of $\theta_i = \theta_c$ which yields $\theta_f = 90^\circ$. For $\theta_i > \theta_c$, the disk will tumble several times before being reinjected at some angle less than θ_c . As this tumbling is deterministic, we expect that the map still exists—but is extremely complicated—for angles $\theta_i > \theta_c$. This part of the map is shown schematically in Fig. 3d as a rapidly fluctuating line. In this sense the map is essentially discontinuous at θ_c .

We now turn to the continuous tumbling regime, observed at very large values of I^* . This regime cannot be analysed in the same way as the previous three, as the disk never comes to rest and hence no values of θ_i are defined. It is not clear if this tumbling behaviour is periodic in nature. The analysis of Maxwell (see, for example, ref. 17) indicates that this might be so; however, direct video observation on lead disks shows some departure from periodicity (Fig. 1d). Also, the velocity of a tumbling disk's centre of mass can vary by large ($\sim 60\%$) amounts from the mean value, in an apparently unpredictable manner¹⁴. These two observations suggest that the rotational period in the tumbling regime is not constant. It is thus possible that the tumbling regime is also characterized by chaotic dynamics, although we have no definitive information on this point. We note that it is possible to access some extremely high values of I^* using paper disks in air, which has a very low value of ρ_f ; to the eye, at least, the rotational period of such disks appears constant. It is therefore possible that at very high values of I^* there is another periodic tumbling regime. □

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Conductivity enhancement in single-walled carbon nanotube bundles doped with K and Br

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Single-walled carbon nanotubes (SWNTs), prepared by metal-catalysed laser ablation of graphite, form close-packed bundles or 'ropes'¹. These rope crystallites exhibit metallic behaviour above 50 K (ref. 2), and individual tubes behave as molecular wires, exhibiting quantum effects at low temperatures^{3,4}. They offer an all-carbon host lattice that, by analogy with graphite⁵ and solid C₆₀ (ref. 6), might form intercalation compounds with interesting electronic properties, such as enhanced electrical conductivity and superconductivity. Multi-walled nanotube materials have been doped with alkali metals⁷ and FeCl₃ (ref. 8). Here we report the doping of bulk samples of SWNTs by vapour-phase reactions with bromine and potassium—a prototypical electron acceptor and donor respectively. Doping decreases the resistivity at 300 K by up to a factor of 30, and enlarges the region where the temperature coefficient of resistance is positive (the signature of metallic behaviour). These results suggest that doped SWNTs represent a new family of synthetic metals.

All experiments were performed on bulk (3 × 8 mm) unoriented samples, or 'mats', using four-point pressure contacts. We estimate that at least 70 vol.% of this material consists of achiral single-walled nanotubes (refs 1, 9, 10) which are predicted to be intrinsically metallic¹¹. The correspondence between rope resistivity ρ and mat resistance is complicated by several factors. First, we can only access some average of tensor components (we expect $\rho_{||} \ll \rho_{\perp}$ by analogy to graphite for which $\rho_a \ll \rho_c$, where the subscripts refer to current flow parallel and perpendicular to the tube axis, or parallel to the graphite *a* and *c* axes respectively). Second, we overestimate ρ if rope-rope contacts are more resistive than the ropes themselves. We expect this factor to be relatively minor owing to the extreme aspect ratio of the rope crystallites and the fact that the highly conducting direction coincides with the longer dimension; the number of rope-rope contacts between voltage probes is expected to be considerably smaller than the number of interparticle contacts in typical powder-pellet resistance measurements². The most important factor is the entanglement of ropes which results in a very low apparent density; and actual area transverse to the current direction is much smaller than the physical cross-section, typically by a factor of 50. We compensate approximately for this porosity by calculating ρ using an effective microscopic area determined from the mass and total length of the mat sample and an assumed microscopic density of 2 g cm⁻³ (between that of graphite and solid C₆₀). For example, a typical mat sample, ~0.2 mm thick if measured with a calliper using light pressure, should weigh ~10 mg if the density was 2 g cm⁻³, whereas the measured mass is only 200 µg.

We measured resistance as a function of time of exposure to Br₂ vapour, using an apparatus similar to that employed for the first doping experiments on solid C₆₀ (ref. 12). A few drops of Br₂ were

initially frozen with liquid nitrogen in the bottom half of a 1-inch-diameter glass vessel equipped with an O-ring seal; the sample and lead frame were suspended from the upper half by the leads and glass-to-metal seals. Figure 1 shows that once the Br₂ was allowed to melt, the resistivity dropped from its initial value, 0.016 Ω cm, to 0.001 Ω cm in less than 4 minutes. Refreezing the Br₂ had little effect, as did opening the vessel and exposing the doped sample to air. Only by heating the sample with a heat gun for 5 minutes did ρ begin to recover. After cooling back to a temperature *T* of 300 K we found a stable plateau at 0.008 Ω cm, half the initial value. Re-doping with Br₂ again decreased ρ to 0.001 Ω cm, and these same limiting values were obtained on repeated cycling. In a separate experiment with a larger sample, we ascertained from weight uptake and loss the average compositions noted in Fig. 1.

We measured $\rho(T)$ on another sample before and after Br₂ doping and undoping, using a wide-temperature-range Joule-Thompson cryostat (Model R2205-23, MMR Technologies, Mountain View, California). Curve a in Fig. 2 shows the behaviour typical of pristine SWNT mats², namely a weakly metallic $\rho(T)$ above a crossover temperature *T*^{*} which for this sample is ~280 K. We have previously presented evidence that *T*^{*} correlates directly with disorder; snag-free single ropes have *T*^{*} ≈ 40 K whereas material with a lower yield of SWNTs has *T*^{*} so high that positive $d\rho/dT$ is never observed. Curve b in Fig. 2 is for saturation doping, after transfer in air from the doping apparatus to the cryostat. In addition to the overall reduction in ρ , positive $d\rho/dT$ persists down to a much lower temperature than in the pristine material, below the limit (90 K) of the apparatus. Heating the sample to 450 K in the cryostat vacuum leads to an increase in ρ , curve c, while maintaining positive $d\rho/dT$.

The results in Figs 1 and 2 suggest the existence of (at least) two different Br populations, weakly and strongly bound, in the doped material. This is again reminiscent of intercalated graphite, in which even after long pumping and heating, some fraction of the intercalate is retained at defect sites in the so-called "residue compound"¹⁵. A second possibility is the existence of a stable phase with intermediate Br concentration, analogous to the staging phenomenon in graphite compounds.

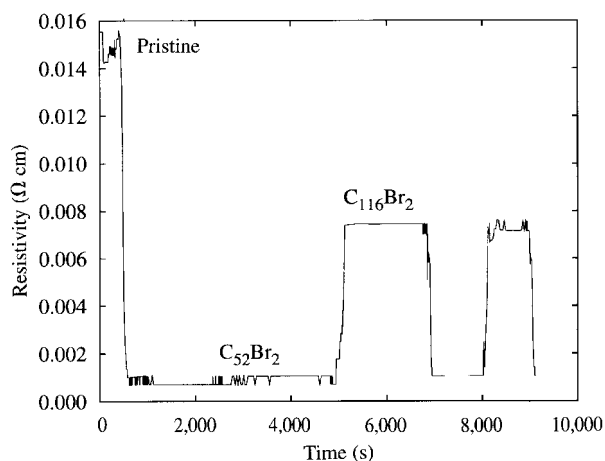


Figure 1 Directionally averaged resistivity (~300 K) of a bulk SWNT sample versus time of exposure to Br₂ vapour. Within 3 min, ρ has dropped from 0.015 to 0.001 Ω cm, with weight uptake (determined in a separate experiment) corresponding approximately to C₅₂Br₂. After 3,000 s, the apparatus was opened to the air, with no change in ρ . After 5,000 s, the sample was heated in air with a heat gun for ~200 s, which increased ρ to 0.008 Ω cm and decreased the Br₂ content as shown. Re-exposing the same sample to Br₂ vapour at 7,000 s returned ρ to its previous minimum value. One additional heating/re-doping cycle is included in the figure.

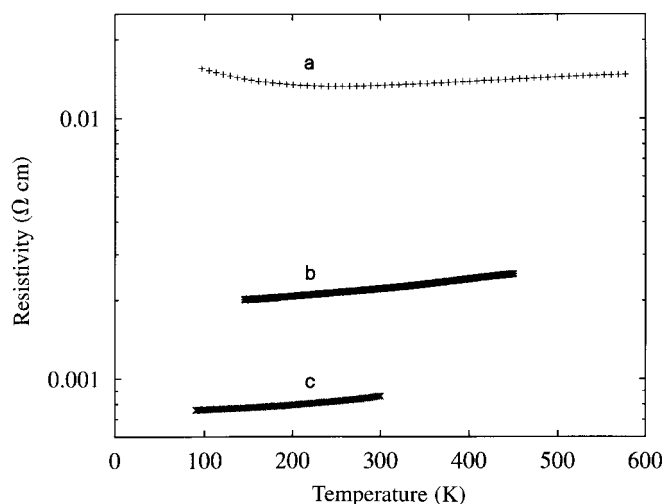


Figure 2 Resistivity versus temperature for a bulk SWNT sample. Curve a, pristine material; curve b, saturation doped with Br₂; curve c, after heating in the cryostat vacuum to 450 K for several hours.

Potassium doping also leads to a large enhancement in conductivity. Potassium metal was distilled into a glass tube containing a mat sample, sealed off, and reacted (473 K for 1–2 days) with a small temperature gradient to avoid coating the mat with K metal. Weight uptakes under these conditions correspond approximately to KC₈. Figure 3 shows a series of $\rho(T)$ measurements on one such sample. Curve a for this pristine sample reveals a lower ρ and T^* than the initial curve in Fig. 1. Curve b was measured after transferring the doped sample in a glove box from the reaction tube into the cryostat. The overall reduction in ρ is a factor of ~ 20 at 300 K, and again T^* is reduced to below our 90 K measurement limit, curve b. Heating overnight to 580 K in the cryostat vacuum yielded curve c, from which we find a smaller $d\rho/dT$ slope with ρ still one-tenth of the pristine value. Finally, curve d resulted from 580 K vacuum anneal for 3 days, with only a small additional resistivity increase. Similar to the Br₂ experiments above and in agreement with Raman data¹³, we find considerable high-temperature stability of the reduction in resistivity. This suggests either strong bonding of part of the dopant at defect sites or the existence of stable intermediate phases; the former is characteristic of graphite compounds⁵ whereas examples of the latter occur in alkali-doped fullerenes⁶. In contrast to Br₂ doping, exposing this sample to air immediately led to a large increase in resistivity to several times greater than the pristine values.

The above results are consistent with Raman scattering data for the same dopants presented in the accompanying report¹³, indicating that for K or Br₂, substantial electron density is respectively added or removed from the host carbon framework. The natural interpretation is that either electron or hole doping accompanies reductive or oxidative intercalation, as in graphite, with enhanced metallic behaviour in either case owing to increased carrier density. It is highly unlikely that the enhanced metallic behaviour results from reactions of K or Br₂ with minor constituents of our SWNT samples—principally amorphous carbon and residual Ni/Co catalyst particles encapsulated by graphitic ‘onions’. Electron microscopy shows that both are widely dispersed and thus unlikely to form a macroscopic conducting path. In separate experiments we determined that Br₂ vapour has only a minor effect on the ‘amorphous’ carbons typically studied for lithium-ion battery applications¹⁴. Furthermore the graphite skin on the Ni/Co particles must be free of defects and discontinuities as it resists attempts to

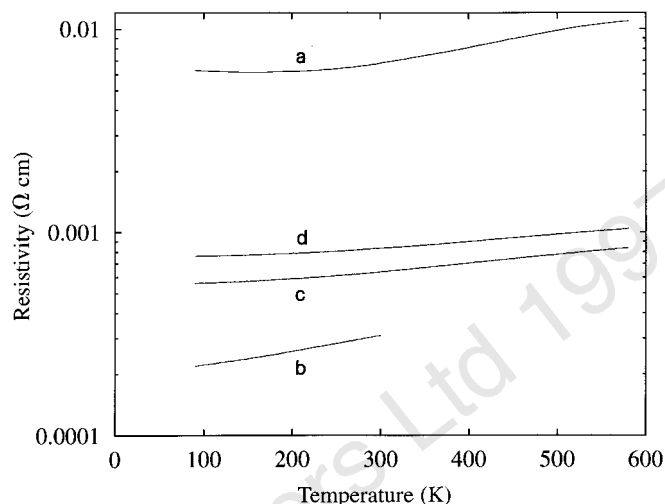


Figure 3 Resistivity versus temperature for a bulk SWNT sample. Curve a, pristine material (different batch than in Fig. 2); curve b, after doping with potassium at 473 K; curve c, after heating in the cryostat vacuum to 580 K overnight; curve d, after 3 d at 580 K.

remove the metal by intercalation followed by acid leaching—material thus treated remains magnetic.

Doping with K or Br₂ also yields important changes in the overall $\rho(T)$ behaviour. The net effect is a reduction in the crossover temperature T^* , above which the sign of $d\rho/dT$ changes from negative to positive. Balents and Fisher¹⁵ conjectured that the crossover in pristine material can be understood as two additive contributions; for example, with T exponents of opposite sign². From Figs 2 and 3, we see that the primary doping effect on $\rho(T)$ is a ‘rigid’ reduction with little or no change in slope, which would cause T^* to increase if the low- T component were unaffected. Furthermore, if $\rho(T)$ in the high- T regime were limited by electron–electron interactions¹⁵, one would expect a doping-induced slope change, or at least important differences between K- and Br₂-doping. Although there appears to be a direct correlation between T^* and disorder², it is hard to imagine that the pristine material is more disordered than the doped material. Measurements below 90 K to probe the effects of doping on the negative slope contribution will be important to understand this effect. X-ray experiments to locate the K and Br dopants are in progress. Also of interest is the possibility of ‘tuning’ the electron transfer so that the Fermi energy coincides with a one-dimensional singularity in the electronic density of states¹⁶, in which case one might be able to overcome the intrinsically weak electron–phonon interaction with a large density of states at the Fermi level to obtain superconductivity. A novel approach, unique to single-walled tubes, would be to exploit the quantum phenomena observed at very low temperatures^{3,4}; doping-induced shifts in conductance peaks versus voltage should be directly related to the displacement in Fermi energy. □

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Evidence for charge transfer in doped carbon nanotube bundles from Raman scattering

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Single-walled carbon nanotubes¹ (SWNTs) are predicted to be metallic for certain diameters and pitches of the twisted graphene ribbons that make up their walls². Chemical doping is expected to substantially increase the density of free charge carriers and thereby enhance the electrical (and thermal) conductivity. Here we use Raman spectroscopy to study the effects of exposing SWNT bundles¹ to typical electron-donor (potassium, rubidium) and electron-acceptor (iodine, bromine) dopants. We find that the high-frequency tangential vibrational modes of the carbon atoms in the SWNTs shift substantially to lower (for K, Rb) or higher (for Br₂) frequencies. Little change is seen for I₂ doping. These shifts provide evidence for charge transfer between the dopants and the nanotubes, indicating an ionic character of the doped samples. This, together with conductivity measurements³, suggests that doping does increase the carrier concentration of the SWNT bundles.

We generate SWNT bundles by a dual pulsed-laser technique¹, which is known to produce a very narrow diameter distribution peaked around the diameter of the (10,10) ('armchair') nanotube (here the indices refer to the diameter and pitch of the tubes). The nanotubes are grouped into aligned bundles of as many as 100–500 tubes, packed into a triangular lattice. The chemical doping of the SWNT bundles was carried out in sealed quartz ampoules using vapour-distilled reactants. Our method parallels those used to intercalate graphite⁴. In the SWNT bundles studied here, the dopant is not expected to lie inside the tube; it may be confined to the interstitial channels found between the tubes in the bundles, or may decorate the entire exterior surface of the SWNTs. If the tubule ends were open, however, it should also be possible to place dopant ions or molecules inside the tubes, as has been done with very small (~1.2 nm inside diameter) multi-wall nanotubes^{5,6}.

The reactant and SWNT sample were positioned at opposite ends of an evacuated ampoule; the reactant vapour travels to the far end of the ampoule where the reaction takes place. The reaction with I₂ and Br₂ were carried out with both the sample and reactant at room temperature; the reaction is quite fast with Br₂ (minutes), whereas I₂ requires several hours to saturate the sample. The alkali-metal reactions were both carried out with the alkali metal and SWNTs at elevated temperatures for 24 h (metal temperature, 120 °C; SWNT temperature, 160 °C). Raman scattering spectra were then measured while the samples remained sealed in their reaction ampoules. All the spectra reported here were measured in the backscattering configuration using 514.5-nm laser excitation. The scattered light was analysed in a Jobin Yvon HR460 single-grating spectrometer equipped with a charge-coupled array detector and a holographic notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI, USA). To avoid laser damage to the sample, all the data were taken at low laser power (2 W cm⁻²) using a cylindrical lens to focus the laser radiation onto the sample (which was a loosely compacted mat of SWNTs). No polarization analyser was used, so that light polarized both perpendicular and parallel to the scattering plane was collected. Figure 1 shows the $T = 300$ K Raman spectra of pristine SWNTs and those doped with the anticipated electron-acceptor (I₂, Br₂) and electron-donor (K, Rb) reagents.

We first discuss briefly the Raman spectrum for pristine SWNTs (Fig. 1, middle spectrum). As discussed previously⁷, the strong peak at 186 cm⁻¹ has been identified with the A_g symmetry radial breathing mode, and the peak at 1,593 cm⁻¹ has been assigned to an unresolved Raman triplet identified with tangential C-atom displacement modes, one of A_g and two with E_g symmetry. These three, nearly degenerate, tubule phonons are related to phonons in the highest-frequency optical phonon branch of graphite which exhibits a zone centre E_{2g} symmetry intralayer mode at 1,582 cm⁻¹. The relative peak intensities of all the Raman-active modes depend critically on the laser wavelength which indicates that the scattering is resonant⁷. For the assignments of the weaker peaks in the pristine spectrum, see ref. 7.

We next address the changes in the Raman spectrum of the pristine tubes due to chemical doping (Fig. 1). The top two spectra in Fig. 1 are for SWNT bundles reacted with typical electron-acceptor dopants (I₂, Br₂) which are expected to transfer electrons from the carbon π states in the tubules to the dopant molecules, creating hole carriers in the SWNTs. The I₂-doping induces the smallest change in the SWNT Raman spectrum. This is perhaps not surprising, as I₂ does not intercalate into graphite. It does diffuse into solid C₆₀ (refs 8, 9), but no convincing evidence for charge transfer with the fullerene lattice has been reported. For the chemically doped SWNTs, we assign the most intense low-frequency and high-frequency modes to the radial and tangential modes, respectively. Iodine-doping of SWNT bundles is seen to slightly increase the radial mode frequency from 186 to 188 cm⁻¹ ($\Delta_r = +2$ cm⁻¹), and slightly decrease the tangential mode frequencies from 1,593 cm⁻¹ in the pristine SWNT to 1,590 cm⁻¹ ($\Delta_t = -3$ cm⁻¹), where Δ_r and Δ_t refer to the change in the radial and tangential mode frequencies on doping. For the case of saturated Br₂-doping, relatively large increases in the radial and tangential mode frequencies are observed: $\Delta_r = +74$ cm⁻¹ and $\Delta_t = +24$ cm⁻¹. We note that the 1,617 cm⁻¹ peak in the Br₂-doped SWNT decreases in frequency to 1,603 cm⁻¹ on refreezing the bromine vapour at the opposite end of the reaction tube; that is, the peak does not shift all the way back to the pristine value at 1,593 cm⁻¹. This suggests the existence of an intermediate-concentration Br₂-doped phase, in agreement with the resistivity studies on Br₂-doped SWNT³. Also in agreement with these resistivity studies³, the Raman spectrum of saturated Br₂-doped SWNT bundles shown in Fig. 1 is recovered again when the Br₂ returns to room temperature and the increased vapour pressure drives the Br₂-SWNT system back to the Br₂-saturated phase.

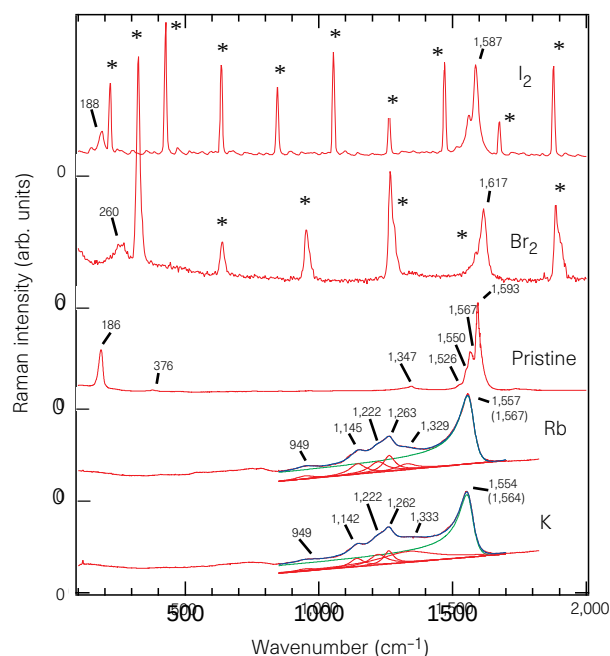


Figure 1 Raman scattering spectra for pristine SWNT bundles reacted with various donor and acceptor reagents. From top to bottom: I_2 , Br_2 , pristine SWNT, Rb and K. The backscattered spectra were taken at $T = 300\text{ K}$ using 514.5-nm radiation. In the spectra for both of the halogen-doped SWNT bundles, a harmonic series of peaks (indicated with an asterisk) is observed, which are identified with the fundamental stretching frequency ω_0 : $\sim 220\text{ cm}^{-1}$ (I_2) and $\sim 324\text{ cm}^{-1}$ (Br_2). The spectra have been scaled so that the strongest SWNT feature appears to have the same intensity. In the vicinity of the strongest high-frequency mode around $1,550\text{ cm}^{-1}$, the Raman spectra for SWNTs doped with K or Rb are fitted with a superposition of Lorentzian functions (red curves) and an asymmetric Breit-Wigner-Fano lineshape (green curve) on a linear continuum; see text. In both cases, the calculated spectrum (blue curve) is nearly superimposed on the data. Peak frequencies are indicated; the values in parentheses are the renormalized phonon frequencies (see text).

The two spectra at the bottom of Fig. 1 are for SWNTs that have reacted with typical electron-donor reagents (K, Rb) which transfer electrons to itinerant carbon π^* states in the SWNTs. As can be seen in the figure, the low-frequency radial breathing mode is not evident in the spectrum. It may have shifted below our lowest detectable frequency ($\sim 100\text{ cm}^{-1}$), or, alternatively, it may have broadened to such an extent that it cannot be detected above the background. A noticeably large, and nearly identical, downshift of all the high-frequency Raman structure is observed for both the K and Rb dopants, even though the Rb atom has a much larger diameter and mass than the K atom. The broad, asymmetric peak at $\sim 1,565\text{ cm}^{-1}$ observed in both alkali-metal-doped SWNT bundles is tentatively identified with a Breit-Wigner-Fano (BWF) interference¹⁰. In solids, this resonance usually involves an interference between Raman scattering from continuum excitations and that from a discrete phonon, provided the two Raman-active excitations are coupled. The degree of coupling ($1/q$) determines the departure of the lineshape from a symmetric Lorentzian function. Similar broad BWF resonances are observed in stage 1 donor graphite intercalation compounds (GICs; for example, MC_8 , where M is K, Rb, Cs), and not in the lower charge transfer, stage 2 donor GICs (for example, MC_{24} , where M is K, Rb, Cs). The positions of the BWF peaks in the stage 1 GICs are slightly lower than those observed here. We assign the BWF peak in the alkali-metal-doped SWNTs to interference scattering between either an electronic or multi-phonon continuum and the highest-frequency tube phonons

which have been softened by electron transfer.

When the coupling between the discrete mode and the continuum is significant, the BWF peak position can be considerably displaced from the frequency of the uncoupled discrete phonon¹⁰. This is referred to as a renormalization of the 'bare' phonon frequency. If the continuum scattering in the SWNTs is electronic in origin, then the degree of frequency renormalization can be an important measure of the electron-phonon interaction. This aspect of the BWF resonance will be addressed elsewhere. The BWF lineshape is given by

$$I(\omega) = I_0 \left\{ 1 + (\omega - \omega_0)/q\Gamma \right\}^2 / \left\{ 1 + ((\omega - \omega_0)/\Gamma)^2 \right\} \quad (1)$$

where I_0 , ω_0 and Γ are, respectively, the intensity, renormalized frequency and broadening parameter. In Fig. 1, we show a fit (blue curve) to the high-frequency experimental spectra for Rb- and K-SWNTs. The solid red curves represent the individual Lorentzian peak shapes used for the peaks at ~ 949 , $1,145$, $1,222$, $1,263$ and $1,329\text{ cm}^{-1}$ and the BWF lineshape (green curve) with its peak at $1,567\text{ cm}^{-1}$. The Lorentzian peaks in the figure are tentatively assigned to charge-transfer-softened modes; they are not observed in the pristine SWNT spectrum. The doping-induced increase in the Raman-activity of these mid-frequency phonons is not understood; it may be related to a breakdown in the pristine nanotube selection rules associated with the doping. The linewidths are also very large, suggesting broadening through either the electron-phonon interactions or inhomogeneous doping. Assignment of these mid-frequency (~ 900 – $1,200\text{ cm}^{-1}$) features must await further work.

BWF lineshapes have been observed previously in doped M_3C_{60} (where M is K, Rb)¹¹ and MC_8 (where M is K, Rb, Cs) GICs⁴. The negative value of $1/q = -0.35$ we observe for the alkali-metal-doped SWNT bundles is about a factor of three smaller than those observed for the broad BWF resonance in MC_8 compounds¹². Similar values of $1/q$ to those found here have been reported for the low-frequency H_g symmetry, intramolecular vibrations in M_3C_{60} (ref. 11).

We now consider if the doping-induced shifts in the SWNT phonon frequencies that we observe are consistent with the results of the Raman studies of GICs (ref. 4), and weight uptake measurements on doped SWNTs by Lee *et al.* in the companion Letter³. We appeal to results of previous studies of charge-transfer-induced changes in the high-frequency intralayer mode (E_{2g2}) in acceptor and donor GICs (for a review, see ref. 4). In these compounds, charge is exchanged between the intercalated layer and the adjacent (or 'bounding') carbon layers. The degree of charge transfer may be defined by the quantity f which is the transferred charge per host C atom. For example, in KC_8 , the charge transfer is essentially complete (the K is almost fully ionized to K^+) and $f \approx 1/8$ (ref. 12). Using stage 1 and stage 2 Raman data from alkali-metal-doped GICs⁴, we estimate the average value $\Delta\omega/\Delta f \approx -140\text{ cm}^{-1}$, where the minus sign indicates that the intralayer mode softens with increasing charge transfer into the π^* band. For the acceptor compounds, we use results from Raman scattering studies of the continuous electrochemical charging of the C layers in stage 1 graphite- H_2SO_4 (ref. 13) from which $\Delta\omega/\Delta f \approx +460\text{ cm}^{-1}$ is obtained; that is, the intralayer mode stiffens with the introduction of holes into the π band.

For Br_2 , which is known to be a weak acceptor (that is, it has a small f value) in a GIC, and using $\Delta\omega/\Delta f \approx +460\text{ cm}^{-1}$, we estimate $f \approx 1/19$, or one free hole per 19 C atoms in the SWNT. However, weight uptake measurements by Lee *et al.*³ indicate that an effective composition $C_{52}Br_2$ has been obtained under similar reaction conditions. Under these assumptions, we must therefore conclude that the acceptor SWNTs exhibit a much larger value of $\Delta\omega/\Delta f$ than observed in GICs. For I_2 -doped SWNTs, the shift of the $1,593\text{ cm}^{-1}$ peak is quite small, consistent with either very weak charge transfer between I_2 and the tube wall or simply a physisorption of dopant on the tube wall. In fact, the frequency decreases slightly, as if the I_2

behaves as a donor, which is not expected.

For the alkali-metal dopants K and Rb, the high-frequency modes show a BWF interference lineshape. As discussed above, this interference introduces a frequency renormalization for the high-frequency modes. Without a detailed analysis involving the spectral shape of the interfering continuum excitations, as we performed earlier for CsC_8 (ref. 10), we cannot separate the two contributions (that is, charge transfer and interference coupling ($1/q$)) to the phonon frequency ω_0 (equation (1)). Consequently, a quantitative statement about the charge-transfer-induced shift in the alkali-metal-doped SWNTs cannot be made at present. Although the one-dimensional nature of the SWNTs is expected to alter the nature of the continuum excitations, the $T \approx 300$ K BWF frequency ω_0 of $\sim 1,567 \text{ cm}^{-1}$ (Rb) and $1,565 \text{ cm}^{-1}$ (K) observed in the doped SWNT bundles is remarkably close to that in the stage 1 MC_8 GICs: $1,547 \text{ cm}^{-1}$ (KC_8) and $1,519 \text{ cm}^{-1}$ (CsC_8) for $T \approx 300$ K. The coupling constants ($1/q$) are, however, a factor of ~ 3 higher in the GICs. We can say that the alkali-metal-doped SWNT Raman spectra qualitatively indicate that the bundles are highly doped, which is consistent with the large weight uptake (effective composition KC_8) observed by Lee *et al.*³.

We have also investigated the temperature stability in vacuum of the alkali-metal-doped SWNT bundles. A series of Raman spectra were taken on a Rb-doped SWNT sample as a function of temperature in the range $24\text{--}380^\circ\text{C}$. The spectra were measured with the Rb-containing end of the evacuated ampoule at room temperature while the sample end of the ampoule was heated to progressively higher temperatures and equilibrated. A frequency decrease, probably associated with the thermal expansion of the SWNTs, and a broadening of the BWF peak, was observed at the highest temperatures. Interestingly, near $\sim 200^\circ\text{C}$, sharp Raman peaks around $1,480$ and $1,590 \text{ cm}^{-1}$ were observed as shoulders on the BWF resonance, suggesting the existence of a metastable phase near 200°C with a different structural arrangement for the Rb ions. At elevated temperatures, these additional peaks disappeared. At the highest temperature (380°C), the Raman spectra suggests that the Rb remains bonded to the tube wall. *In situ* studies of Br_2 uptake at $T \approx 300$ K in these SWNT bundles also reveal an intermediate-concentration phase. In this case, the high-frequency peak at $1,593 \text{ cm}^{-1}$ was found to quickly (<5 min) increase in frequency on exposure to bromine vapour and attain an intermediate value ($1,603 \text{ cm}^{-1}$); after some time, the frequency changed rapidly to the saturation-doped value ($1,617 \text{ cm}^{-1}$). □

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Seismological evidence for three-dimensional melt migration beneath the East Pacific Rise

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The extent to which crustal processes along mid-ocean ridges are controlled by either the pattern of mantle upwelling or the mode of magma injection into the crust is not known. Models of mantle upwelling vary from two-dimensional, passive flow¹ to three-dimensional, diapiric flow^{2–4}. Similarly, beneath a ridge segment bounded by tectonic offsets, crustal magma chambers may be replenished continuously along the ridge^{5–7} or at a central injection zone^{2–4} from which magma migrates towards the segment's ends. Here we present tomographic images that reveal the seismic structure and anisotropy of the uppermost mantle beneath the East Pacific Rise. The anisotropy is consistent with two-dimensional mantle flow diverging from the rise, whereas the anomalous isotropic structure requires a three-dimensional but continuous distribution of melt near the crust–mantle interface. Our results indicate that crustal magma chambers are replenished at closely spaced intervals along-axis and that crustal systems inherit characteristics of scale from melt transport processes originating in the mantle.

The site of our investigation lies midway along a 100-km-long section of the East Pacific Rise (EPR) bounded by the $9^\circ 03' \text{N}$ overlapping spreading centre and the Clipperton transform, a comparatively well studied section of oceanic ridge (Fig. 1). Its broad axial summit², axial-magma-chamber (AMC) reflector^{8,9}, crustal low seismic-wave velocity^{6,10}, and low-Q zone¹¹ are characteristic of a fast-spreading ridge that is magmatically robust. Apart from the overlapping spreading centre, the rise axis is continuous except for a series of fine-scale morphological discontinuities where the local trend of the rise changes abruptly by $10^\circ\text{--}15^\circ$ (refs 2, 5, 12). Such deviations from axial linearity ('devals') segment the rise at intervals of 10–20 km and show no off-axis trace^{2,5}. Devals are thought to demarcate axial hydrothermal systems¹², petrologically distinct lava^{5,13} and the axial continuity of the AMC reflector⁹. Crustal tomography^{6,11,14} reveals an axially varying seismic structure that also correlates with the segmentation observed in sea-floor morphology and geology. As comparatively little is known about the structure at sub-crustal depths, the origin of this segmentation is enigmatic. It may derive from magmatic processes occurring in the mantle or from tectonic and hydrothermal processes confined to the crustal carapace.

Our tomographic analysis includes delay times from 1,362 crustal and 200 P_n (mantle) refractions (Fig. 2a). Examination of Fig. 2b, which shows delay times for sets of receivers on opposing sides of the rise, suggests that the data are consistent with both seismic heterogeneity and anisotropy within the uppermost mantle. To permit imaging in anisotropic media, we modified our tomographic method¹⁴ to allow an azimuthally anisotropic upper mantle, a symmetry system in accord with previous studies of oceanic P_n arrivals (see review in ref. 15). The velocity of P_n waves for this symmetry system follows a $\cos(2\theta)$ relationship, where θ is the azimuth of energy propagation¹⁵. We define a slowness model (slowness is the inverse of velocity) as

$$u'(r, \theta_p; u(r), a(r), \theta(r)) = \frac{u(r)}{1 + \frac{a(r)}{2} \cos[2(\theta(r) - \theta_p)]} \quad (1)$$

where $u(\mathbf{r})$ is the average slowness at nodal position $\mathbf{r}$, $a(\mathbf{r})$ is the percentage anisotropy defined as $(v_{\max} - v_{\min})/v_{\text{average}}$, where v is P-wave velocity, $\theta(\mathbf{r})$ is the azimuth of the fast direction for P waves, and θ_p is the azimuth of energy propagation. With the exception that the slowness model is defined by equation (1), we follow a previous implementation of the shortest-path technique¹⁴ to calculate ray paths and travel times through three-dimensional models that explicitly include sea-floor relief. The inverse problem is regularized by minimizing a stochastic penalty function and model roughness. Prior uncertainties in model parameters were 10% in $u(\mathbf{r})$, 10% in $a(\mathbf{r})$ and 8° in $\theta(\mathbf{r})$, and the decay length of the roughness operator was set at 125% of the interval between

perturbational nodes (see ref. 14 for details). From a one-dimensional starting model¹⁰, tomographic inversions first calculated the best-fitting two-dimensional (axially invariant) model and then used this result to find the best fitting three-dimensional model. All inversions included several iterations of the forward and inverse problems to incorporate ray-bending effects.

The results of several tomographic analyses are presented in Fig. 3a–d. Images are shown for three inversions where the percentage of anisotropy was fixed at 0%, 6% and 12%, and the tomographic analyses determined the azimuth of anisotropy and perturbations to the isotropic component of velocity (Fig. 3a–c). Figure 3d shows the results for an inversion where all parameters, including percentage

Figure 1 Sea-floor bathymetry and geometry of the seismic tomography experiment. Bathymetry is contoured at 100-m intervals, grey shade changes every 200 m; the 2,500- and 2,700-m contours are labelled. An array of 15 ocean-bottom receivers, 7 near- and 8 off-axis instruments (filled circles and squares, respectively), recorded data from over 400 explosive sources located within the inner box and from 90 shots located along two off-axis rise-parallel lines (small open circles). The inner box encompasses the region of previous crustal imaging, whereas the region of tomographic analysis of this study is shown by the outer box. The aperture of our experiment encompasses a 12-km-long linear rise segment, defined by two deviations from axial linearity ('devals') near 9° 28' N and 9° 35' N, and portions of adjacent segments to the north and south. Mantle refractions (P_n), observed as first-arrivals for source-receiver paths connecting the two lines located 20 km off-axis, sample structure within ~4 km of the Moho along a 30-km-long section of the rise. As the crustal legs of the P_n paths are positioned off-axis, this phase is not affected by the axial magma chamber and any delay-time anomaly is the result of variation in the structure of the off-axis crust or the uppermost mantle beneath the rise. Crustal phases constrain the structure of the upper 3 km of the crust beneath the source and receiver positions.

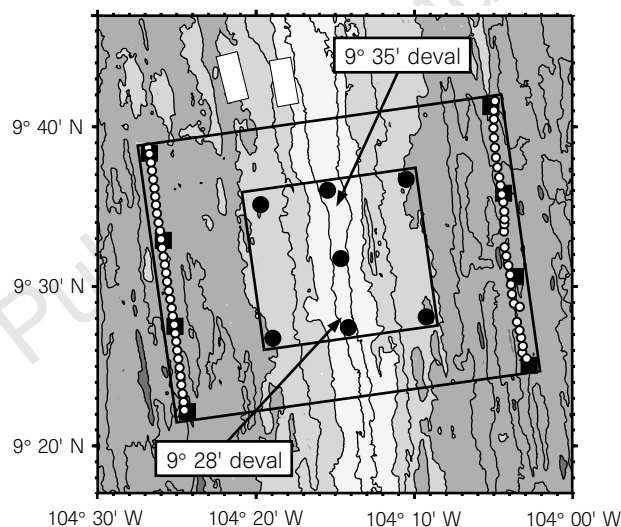
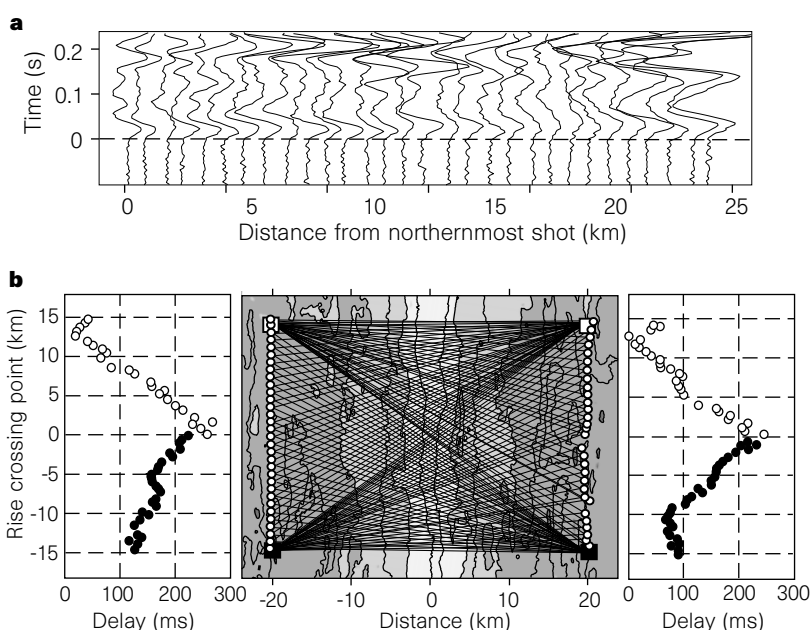


Figure 2 a, Record section showing seismograms from the westernmost sources as recorded by a receiver in the southeastern corner of the experiment (see Fig. 1). Traces are plotted by distance from the northernmost shot and are aligned at the P_n arrival; sampling interval is 4 ms. Source-receiver range along this line varies from 40 to 50 km; throughout this distance the signal-to-noise ratio and the trace-to-trace coherency are both good. P_n arrivals are identifiable to within 10–15 ms. Onset time and uncertainties are assigned by an automatic picking routine¹⁴. The r.m.s. uncertainty in the P_n data alone is 11 ms and the r.m.s. uncertainty in the P and P_n data combined with the experimental uncertainty (see ref. 14) is 9 ms. **b**, Travel-time delays, relative to a one-dimensional model, for four of the off-axis receivers and associated source-receiver pairs. Travel-time delays observed at the northernmost receivers (open circles) show a systematic increase of 250 ms towards the south. If this trend were entirely the result of variation in crustal structure near the source positions, then a matching signal would be predicted for a different pair of receivers recording the same sources. For a pair of receivers to the south (filled circles), such a signal is not observed, indicating that the source of the anomaly is located in the mantle. The delay-time anomaly is less for rise-perpendicular paths, a pattern consistent with seismic anisotropy when the fast axis is aligned with the spreading direction. Features indicative of axis-parallel heterogeneity are a delay-time signal that varies at several wavelengths and that, for rise-perpendicular paths, differs from north to south by 100 ms, with northern paths generally faster (smaller delay) than southern paths.



of anisotropy, were free to vary. The images of seismic heterogeneity are similar, particularly for models that include a component of anisotropy. For the results in Fig. 3, the final root-mean-squared (r.m.s.) residuals for the delay-time data were similar (~ 12 ms; a normalized variance reduction of 95% relative to the starting model). For the P_n data alone, a three-dimensional model achieves a 95% variance reduction (r.m.s. decreases from 87 to 14 ms), whereas the best-fitting two-dimensional model explains $< 50\%$ of the data variance (r.m.s. decreases from 87 to 44 ms). The magnitude of seismic anisotropy is not well constrained, a result of the limited azimuthal sampling ($< 40^\circ$) for a station that records P_n arrivals. A comparison of the images (Figs 3a–c) shows that anisotropy trades off primarily with the average upper-mantle velocity, with increasing anisotropy requiring a lower average velocity; an increase in percentage anisotropy results in faster propagation across the rise, whereas a lower average velocity has the opposite effect. We cannot choose a preferred model on the basis of data misfit alone. However, only models with 6–7% anisotropy (Fig. 3b or d) yield an average sub-axial P-wave velocity (7.3 km s^{-1}) consistent with the results of previous seismic refraction experiments^{10,11} performed in this area. Our preferred solutions (Fig. 3b and d) thus include a significant component of seismic anisotropy (6–7%) with the fast axis rotated $2\text{--}3^\circ$ anticlockwise with respect to the predicted spreading direction¹⁶.

We have performed a number of resolution tests to confirm that the P_n data require significant along-axis variation in sub-Moho structure. Synthetic tests (Fig. 3e–h) indicate that the available ray set reconstructs the approximate shape and magnitude of anomalous structures. Owing to the spatial smoothing operators,

however, the amplitude of a reconstructed anomaly trades off with its width such that the peak magnitude of a velocity perturbation may be underestimated by 15–20% (in terms of absolute velocity the difference is only $\sim 1\text{--}3\%$). Additionally, our results are effectively unchanged by expected variations in crustal thickness. A seismic-reflection study reports that the crustal thickness at the southern end of our experiment may be 1 km greater than at the northern end¹⁷. Such an increase in crustal thickness does not change the along-axis segmentation of the anomalous sub-Moho structure, though it does impart a linear gradient in velocity from north to south. Cross-axis variations in crustal thickness are minimal in this area^{8,17} and cannot explain the P_n delay-time anomaly. To quantify the vertical sensitivity of the P_n data, inversions were conducted for starting models similar to that in Fig. 3b and d, except that the deeper portions of the sub-axial anomaly were selectively removed. Several such inversions reconstructed the lowermost portions of the anomaly, confirming that the P_n data require a low-velocity region that is at least 4 km thick. This result is in agreement with the Fresnel zone of P_n arrivals, which at mantle depths are averaging structure within 2 km of the infinite-frequency ray path. We conclude that the tomographic image recovers along-axis variations in bulk properties averaged over the upper 4 km of the mantle.

A perspective view of the preferred model, along with the sea-floor relief, is shown in Fig. 4. The most prominent feature is the axially continuous region of anomalously low velocities. Directly beneath the rise axis the P-wave velocity, relative to off-axis upper-mantle velocities, is reduced by up to 12% near the experiment's centre and varies along-axis by 6–8% over distances of a few

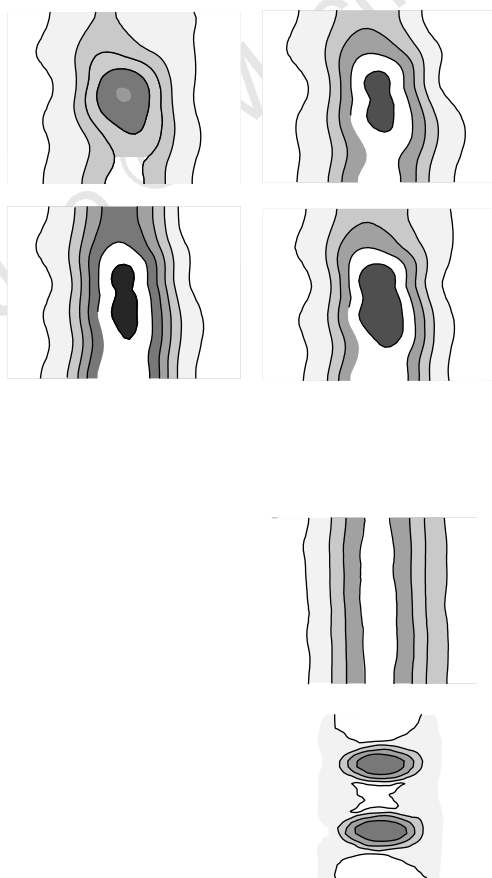


Figure 3 a–d. Results of tomographic analyses for different assumptions of seismic anisotropy. Map-view sections are taken at 8 km depth beneath the sea floor; images between 7 and 10 km depth are similar. Perturbations are shown relative to 8.2 km s^{-1} ; the grey scale changes at intervals of 0.2 km s^{-1} , from 0.0 to -1.2 km s^{-1} . The percentage anisotropy and orientation of the fast direction, with respect to the x-axis, are indicated in the box at the top right of a panel. The area of the sections corresponds to the outer box in Fig. 1; the y-axis is aligned with the regional trend of the EPR. For **a–c**, the magnitude of the anisotropy was fixed at 0%, 6% and 12%, respectively, and the tomographic analysis determined the direction of anisotropy and isotropic component of velocity. **d**, Results when all parameters (magnitude and orientation of anisotropy and isotropic component of velocity), were allowed to vary. For ray tracing, the seismic velocity model was parametrized within a $42 \times 31 \times 11 \text{ km}^3$ volume at intervals of 250 m and 200 m in the horizontal and vertical directions, respectively. In comparison with models where analytical solutions are available, this parametrization yields a standard deviation of 3 ms in the estimate of a P_n travel time (for all ranges and azimuths); further improvements in the accuracy of ray tracing do not affect the results of tomographic imaging. Our inversion method uses separately defined perturbational models for the isotropic velocity and anisotropy parameters. The perturbational model of isotropic velocity is defined at 1-km intervals in the horizontal directions and 0.5-km intervals in the vertical direction, except in the upper 3 km of the model where the vertical spacing is 0.2 km. The depth-dependent perturbational model for anisotropic parameters is defined within the Moho transition zone and the uppermost mantle at 1-km intervals. **e–h**, Tests of resolution. Synthetic travel-time data for two- and three-dimensional structures (**e** and **g**) were inverted using the procedure applied to actual data; random noise (zero mean, 10-ms standard deviation) was added to the data. The two-dimensional test model, **e**, includes an axis-parallel, low-velocity region and 5% seismic anisotropy. The reconstruction, **f**, demonstrates that the ray set does not introduce any significant three-dimensional structure. The three-dimensional synthetic model, **g**, includes two regions of anomalously slow velocities. The lateral velocity contrast of 1 km s^{-1} results in significant diffraction of energy around the anomalies and thus an underestimate of their spatial dimensions (**h**). However, the location, approximate dimensions, and peak amplitude of the low-velocity perturbations are recovered.

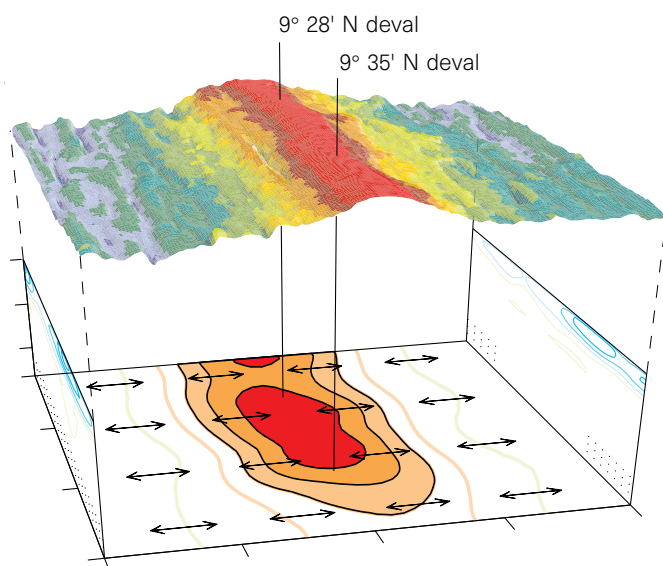


Figure 4 Sea-floor bathymetry and results of tomographic imaging shown in perspective view; solution is that of Fig. 3d. Perturbations in seismic velocity are shown relative to a one-dimensional model¹⁰ (8.2 km s^{-1} in the mantle) and contoured at intervals of 0.2 km s^{-1} . The vertical sections are located $\pm 20 \text{ km}$ from the rise axis; the horizontal section is at 8 km depth beneath the sea floor. The fast axis for the azimuthal P-wave anisotropy is depicted by the arrows. Vertical exaggeration of the bathymetry is five times and the locations of devals are shown by vertical lines. In the horizontal section, the filled areas represent the region predicted to contain $>1\%$ melt. The magnitude of the upper-mantle anomaly varies along-axis by more than a factor of 2 and the anomaly is clearly segmented, with the greatest perturbation (-1.0 km s^{-1}) located between the $9^\circ 28' \text{ N}$ and $9^\circ 35' \text{ N}$ devals. The upper-mantle anomaly lessens significantly in magnitude northward of the $9^\circ 35' \text{ N}$ deval and as far as the edge of our experiment aperture. This portion of the anomaly underlies a section of the rise that shows no evidence of active hydrothermal venting¹² and exhibits an AMC reflector that is about half the width of that to the south of the $9^\circ 35' \text{ N}$ deval⁹. Near the $9^\circ 28' \text{ N}$ deval, the anomaly again decreases in magnitude, before increasing towards the southern limit of the image. These along-axis variations in the magnitude of the upper-mantle anomaly have a striking correlation with the segmentation of sea-floor morphology^{2,5,12} and previously reported crustal images^{6,14}.

kilometres (down to 4% north of the $9^\circ 35' \text{ N}$ deval). The average cross-axis width of the anomaly ($\Delta v_p < -0.2 \text{ km s}^{-1}$, where v_p is P-wave velocity), nearly 18 km , is more than twice the width of the low-velocity volume observed in the crust (see Fig. 3 in ref. 6).

It is reasonable to assume that, at mantle depths, anomalously low seismic velocities are due to elevated temperatures and the presence of partial melt. As our experiment was removed from offsets in the rise that could influence the mantle thermal structure, the observed rise-parallel heterogeneity in seismic velocity is consistent with a region of partial melt that is variable along axis. Estimates of the absolute melt fraction depend on the cross-axis thermal structure. Within 10 km of the rise, thermal models^{18,19} predict maximum sub-Moho temperature differences of $<200^\circ \text{C}$, corresponding to a cross-axis velocity reduction of $<1\text{--}4\%$ (1% , ref. 20; 4% , ref. 21). Given these values, our results are compatible with a region of partial melt that is axially continuous. For film-like inclusions (aspect ratios of $0.1\text{--}0.01$), the range in melt fraction would be $1\text{--}5\%$; this range would double if melt is stored along grain boundary triple junctions^{22,23}. These estimates represent an average on the scale of the seismic wavelength and are in general agreement with values inferred from potential field data (see, for example, refs 24, 25).

The tomographic images of velocity structure at $9^\circ 30' \text{ N}$ (Fig. 4 and refs 6, 11, 14) indicate that mantle and crustal magmatic processes are segmented on a similar length scale. We suggest that the rise between the $9^\circ 28' \text{ N}$ and $9^\circ 35' \text{ N}$ devals overlies mantle that is at present delivering greater amounts of melt to sub-crustal and crustal reservoirs. As volcanic and hydrothermal processes are strongly linked to magmatic processes, we interpret the observed segmentation in crustal structure^{6,11,14}, axial morphology^{2,5,12}, and the spatial distribution of hydrothermal vent fields¹² as a consequence of focused ascent of magma at mantle depths. For a section of the EPR bounded by tectonic offsets, such as transform faults or overlapping spreading centres, several regions of enhanced melt supply from the mantle to the crust are predicted to exist along-axis. □

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Nearly synchronous climate change in the Northern Hemisphere during the last glacial termination

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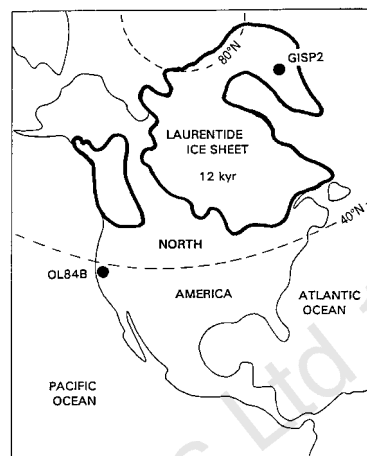
The climate of the North Atlantic region underwent a series of abrupt cold/warm oscillations when the ice sheets of the Northern Hemisphere retreated during the last glacial termination (17.7–11.5 kyr ago). Evidence for these oscillations, which are recorded in European terrestrial sediments as the Oldest Dryas/Bølling/Older Dryas/Allerød/Younger Dryas vegetational sequence^{1,2}, has been found in Greenland ice cores^{3,4}. The geographical extent of many of these oscillations is not well known^{5,6}, but the last major cold event (the Younger Dryas) seems to have been global in extent^{7–10}. Here we present evidence of four major oscillations in the hydrological balance of the Owens basin, California, that occurred during the last glacial termination. Dry events in western North America occurred at approximately the same time as cold events recorded in Greenland ice, with transitions between climate regimes in the two regions taking place within a few hundred years of each other. Our observations thus support recent climate simulations which indicate that cooling of the North Atlantic Ocean results in cooling of the North Pacific Ocean¹¹ which, in turn, leads to a drier climate in western North America¹².

Owens Lake is located in the Great Basin of the western United States between the central Sierra Nevada and the Inyo-White mountains. Maximum precipitation along the Sierra Nevada is associated with the annual north–south progression of the polar jet stream, with cool-season orographic precipitation from North Pacific sources supplying >99% of the runoff reaching the Owens basin^{13,14}.

Core OL84B was obtained from the Owens basin in 1984 using a modified Livingstone piston corer (Fig. 1)¹⁵. A series of accelerator mass spectrometry (AMS) ¹⁴C dates on bulk organic carbon were used to set the chronology of the core section used in this study (Fig. 2). The ¹⁴C-based records of Owens Lake were converted to calendar years using ²³⁰Th–²³⁴U and ¹⁴C ages of corals^{16–19}. The ¹⁴C data indicate sediment hiatuses at 2.25 and 9.20 m. The upper surface of the 9.20-m hiatus contains features indicating desiccation and subaerial exposure, including a lag deposit (1–3 mm thick) of frosted quartz grains. The 2.25-m hiatus is marked by a coarse sand. Sediments examined in this study are thus bounded by two desiccation events that occurred ~18.3–15.8 and ~6.7–4.5 kyr ago (Fig. 3)²⁰. The younger hiatus, previously noted in a core taken from higher elevation in the Owens basin²¹ seems to have occurred at about the same time as hiatuses noted in cores from the Mono Lake basin²². The older hiatus appears to coincide with major declines in the levels of Mono Lake and Lake Lahontan^{23,24}.

To determine the degree of variability in the hydrological balance of Owens Lake between 15.8 and 6.7 kyr, we analysed a continuous

Figure 1 Map of North America showing locations of sediment core OL84B and ice core GISP2.



set of 120 samples for $\delta^{18}\text{O}$ and total inorganic carbon (TIC) (Fig. 3). Each sample integrated ~60 yr of time and was repeatedly washed with distilled–deionized water to remove soluble salts. The isotopic analyses were done on the TIC fraction.

In lakes that oscillate between closed and open hydrological states, $\delta^{18}\text{O}$ and TIC values tend to decrease with decreasing residence time of water in the lake basin²⁰. The $\delta^{18}\text{O}$ value of a lake is a function of the overflow:inflow ratio. When this ratio approaches unity, the $\delta^{18}\text{O}$ value of a lake approaches the $\delta^{18}\text{O}$ value of inflow; when this ratio approaches zero, the $\delta^{18}\text{O}$ value of lake water becomes highly enriched owing to evaporative concentration of the heavy isotope of oxygen (¹⁸O) in lake water. For Owens Lake, calcites precipitated during high overflow:inflow conditions would have $\delta^{18}\text{O}$ values approaching ~15‰ (relative to the VSMOW standard); in contrast, calcites precipitated during hydrological closure would have $\delta^{18}\text{O}$ values approaching ~30‰ (ref. 20).

During overflow, losses of Ca^{2+} (and CO_3^{2-}) increase with increase in the overflow:inflow ratio; therefore, if the flux of detrital silicates remains roughly constant, the TIC fraction becomes a proxy for change in hydrological balance. During hydrological closure, all Ca^{2+} that reaches a lake eventually precipitates as CaCO_3 . The TIC fraction in a closed lake tends to decrease with increasing lake size because the flux of suspended sediment increases exponentially with increasing discharge to the lake, diluting the carbonate precipitate²⁵. Glaciation of a lake basin's catchment area also can markedly increase the flux of detrital silicates to a lake, completely masking the TIC signal²⁰.

The $\delta^{18}\text{O}$ data obtained in this study indicate four extremely dry (closed-basin) intervals (D_1 to D_4) that occurred between 15.8 and 6.7 kyr (Fig. 3). The older three dry intervals are centred at 15.1, 13.2 and 12.2 kyr ago; the youngest dry interval (D_4) marked the beginning of the Holocene (11.3 kyr) and extends to Desiccation II. The presence of prismatic cracking in the reddish D_3 interval suggests the existence of a soil formed during subaerial exposure of lake sediments. Relatively wet intervals (W_1 to W_4) precede each of the dry events. Interval W_1 was not recorded in the $\delta^{18}\text{O}$ data set because the overflow:inflow ratio was too high to permit saturation with CaCO_3 . Interval W_2 contains two secondary peaks (W_{2a} and W_{2b}).

Although not amplitude-locked, the TIC data parallel oscillations in $\delta^{18}\text{O}$ throughout the entire time period (Fig. 3). This parallelism indicates that detrital silicates (glacial rock flour) did not obscure the TIC signal, supporting other studies which indicate that the Sierras were essentially deglaciated by 15–14 kyr ago^{26,27}. The amplitude of TIC variability increases between 8.8 and 6.7 kyr

ago: this suggests that maxima in TIC were caused by carbonate precipitation in extremely shallow lakes. Under these conditions, carbonate precipitation masked the input of detrital silicates whose flux decreased with decreasing inflow of the Owens River. The time of transition to shallow oscillating conditions (8.8 kyr) was coeval with the rapid disappearance of Early Holocene woodlands from the Chihuahuan, Sonoran and Mojave deserts in the southwestern United States ~9 kyr ago²⁸. This change to a drier type of vegetation also indicates an increase in aridity of the southwestern United States after 9 kyr.

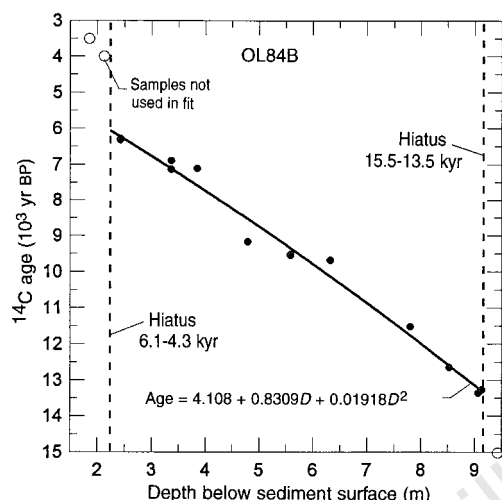


Figure 2 Radiocarbon-age model for core OL84B between depths of 2.25 and 9.20 m. Filled circles indicate ^{14}C analyses on bulk organic carbon used in construction of the age model. Vertical dashed lines indicate location of hiatuses in sedimentation. All age data (kyr) presented in the text of this Letter are in calendar years.

The oscillatory behaviour of TIC was terminated by Desiccation II, which lasted from ~6.7 to 4.5 kyr ago (Fig. 3). Transition to wetter conditions after 4.5 kyr at Owens Lake is consistent with pollen data from meadows in the Sierra Nevada that indicate a transition at ~4.7 kyr to peat and other plant types that require abundant soil moisture²⁹.

Pollen was extracted from 17 sediment samples from OL84B to determine if rapid changes in the hydrological balance of Owens Lake also were reflected in the vegetation community that surrounded the lake. When the climate was wet and Owens Lake occupied most of its basin, juniper (*Juniperus*) or sagebrush (*Artemisia*) should have composed much of the nearby vegetation. When the climate was dry and Owens Lake was small, the Chenoam (*Chenopodiaceae* and *Amaranthus*) group—which includes drought-tolerant salt-resistant desert taxa—should have populated the saline playa abandoned by the retreating lake.

The pollen data indicate that *Juniperus* was abundant between 15.6 and 14.6 kyr ago; thus, it was very wet during the period that followed Desiccation I. After 15.0 kyr, *Juniperus* declined rapidly and then recovered somewhat during subsequent wet phases W_3 and W_4 (Fig. 3). Peaks in the *Artemisia*/Chenoam (A/C) ratio, which indicate relative wet conditions, occurred during minima in the $\delta^{18}\text{O}$ and TIC records (Fig. 3). Thus all three proxies of climate variability yield a consistent picture of oscillations in the hydrological balance of Owens Lake.

Comparison of the $\delta^{18}\text{O}$ record of change in the hydrological balance of Owens Lake with the GISP2 record of the Oldest Dryas/Bølling/Older Dryas/Allerød/Younger Dryas and Holocene cold/warm oscillations indicates a remarkable degree of similarity in the number, duration, and timing of climate regimes in both records between 17.0 and 11.2 kyr ago (Fig. 3). There are five North Atlantic cold regimes centred at 16.4, 14.8, 14.0, 13.2 and 12.3 kyr ago and five dry regimes in western North America centred at ~16.9, 15.1, 14.2, 13.2 and 12.2 kyr ago. Although the errors associated with our age model (± 500 yr; Fig. 2) make it impossible to demonstrate absolute synchronicity between the two records, it is clear that both

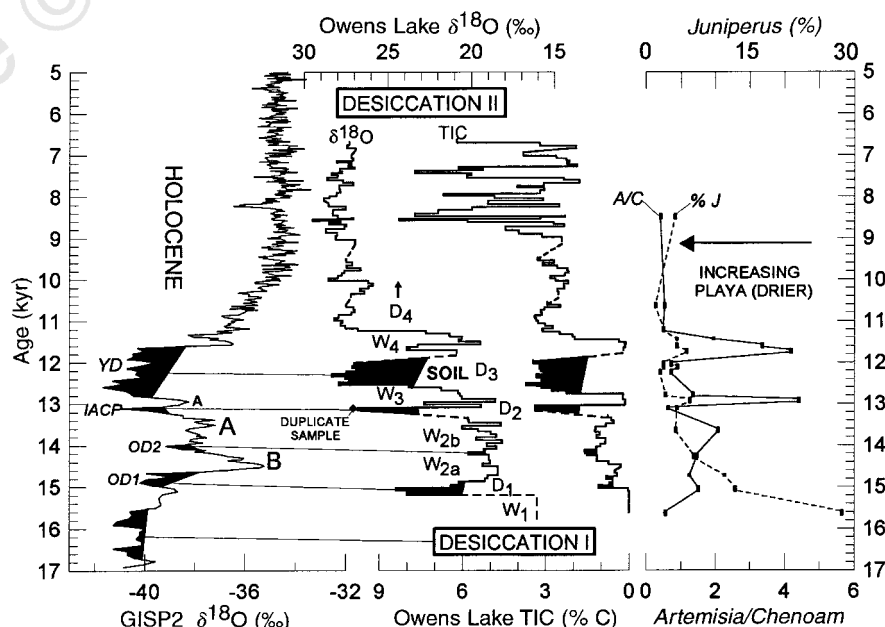


Figure 3 $\delta^{18}\text{O}$, total inorganic carbon (TIC) and pollen records for core OL84B. D_1 to D_4 are relatively dry intervals; W_1 to W_4 are relatively wet intervals. The timing of the Oldest Dryas (OD1)/Bølling (B); Older Dryas (OD2)/Allerød (A); Younger Dryas (YD)/Holocene boundaries and the Inter-Allerød Cold Period (IACP) are based on ice-layer counts from GISP2³⁴. Cold intervals in the ice-core record and dry periods in the Owens Lake record are indicated by black intervals. Note that

the ^{14}C age model for core OL84B is accurate to only a few hundred years. This implies that the $\delta^{18}\text{O}$ record from OL84B could lead or lag the $\delta^{18}\text{O}$ record from GISP2; that is, oscillations present in both cores cannot be demonstrated to be synchronous. In the pollen record shown, A indicates *Artemisia*; C, Chenoam (*Chenopodiaceae* + *Amaranthus*) and J, *Juniperus*.

records attest to a similar number of large and abrupt climate oscillations during the last glacial termination. We argue that, in general, Atlantic cold events (for example, the Younger Dryas occurred during dry intervals in western North America (for example, D₃); also, warm events in the Atlantic region (for example the Bølling) occurred during wet intervals in western North America (for example, W_{2a}). The last wet phase (W₄) occurred during the last Greenland warm peak.

With the advent of the Holocene, linkage of the climate regimes of the North Atlantic and western North America weakened and perhaps disappeared. Before the Holocene, relatively dry conditions occurred in western North America when the North Atlantic region was relatively cold. During the Early and Middle Holocene this relationship was reversed; that is, western North America was relatively dry and the North Atlantic region was relatively warm.

The duration, timing and similar number of climate oscillations in western North America and the North Atlantic region, indicated by this and other studies²⁰, suggests a climate-change link during the last glacial termination throughout at least part of the Northern Hemisphere. Errors inherent in our age model do not allow us to completely rule out an oceanic linkage; however, recent climate simulations more strongly support the concept of atmospheric forcing^{11,12}. In agreement with these studies, we suggest that oscillations in wetness and temperature in western North America were linked to oscillations in the strength and pattern of the North Atlantic thermohaline circulation through its effect on sea surface temperature and atmospheric water content. Rapid climate oscillations in the North Atlantic regions have been attributed to sudden changes in the rate and location of thermohaline overturn^{30–33}. We propose that cooling of the North Atlantic, resulting from a decrease in thermohaline circulation, caused a downstream cooling of the North Pacific¹¹, which in turn decreased the temperature and moisture content of air passing over the middle latitudes of western North America¹². □

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Landscape ecology of algal symbionts creates variation in episodes of coral bleaching

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Reef-building corals are obligate, mutualistic symbioses of heterotrophic animals and phototrophic dinoflagellates (*Symbiodinium* spp.)¹. Contrary to the earlier, widely accepted belief that corals harbour only one symbiont, we found that the ecologically dominant Caribbean corals *Montastraea annularis* and *M. faveolata* can act as hosts to dynamic, multi-species communities of *Symbiodinium*. Composition of these communities follows gradients of environmental irradiance, implying that physiological acclimatization^{2–4} is not the only mechanism by which corals cope with environmental heterogeneity. The importance of this diversity was underlined by analysis of a natural episode of coral bleaching. Patterns of bleaching could be explained by the preferential elimination of a symbiont associated with low irradiance from the brightest parts of its distribution. Comparative analyses of symbionts before and after bleaching from the same corals supported this interpretation, and suggested that some corals were protected from bleaching by hosting an additional symbiont that is more tolerant of high irradiance and temperature. This 'natural experiment' suggests that temporal and spatial variability can favour the coexistence of diverse symbionts within a host, despite the potential for destabilizing competition among them^{5,6}.

The corals *Montastraea annularis* and *M. faveolata* each host three distantly related taxa⁷ of the dinoflagellate genus *Symbiodinium*, denoted A, B and C, that are identified by restriction-fragment length polymorphisms (RFLPs) in genes encoding small ribosomal RNA (srRNA)⁷. A and B are common in shallow-water corals (high-irradiance habitats), whereas C predominates in deeper corals (low-irradiance habitats). Mixed samples A + C and B + C, common at

intermediate locations⁷, suggest that symbionts may actually exist as complex communities that track differences in irradiance within a colony².

To test this hypothesis we sampled four locations in each of 46 colonies (Fig. 1). All *M. faveolata*, and all but one colony of *M. annularis*, yielded two or three types of symbionts. As predicted, *Symbiodinium* A and B dominated locations with higher, downwelling irradiance (communities 1 and 2, unshaded colony tops), and C dominated locations of lower irradiance (communities 3 and 4, colony sides and shaded colony tops) ($P < 0.001$; χ^2 test). These patterns of intra-colony zonation largely disappear at slightly greater depths (8–11 m in *M. annularis*, and 6–12 m in *M. faveolata*), where *Symbiodinium* C is predominant overall⁷. As before⁷, *Symbiodinium* A was more common in *M. faveolata* than in *M. annularis*.

Analyses at a finer scale (~1 cm) confirmed that symbionts occupy distinct but overlapping habitats (Fig. 2). Unshaded columns of *M. annularis* create a localized gradient of low (on the side,

no downwelling) to high (on the top, full downwelling) irradiance, which we sampled along transects. At intermediate depths (3–7 m) this gradient coincides with the transition from *Symbiodinium* C to B, B + C, or A (Fig. 2a–d). Analyses of shallower (1–2 m) and deeper (9–12 m) corals (Fig. 2d) show that depth⁷ and intracolony zonation of the symbionts occur in parallel. These consistent patterns strongly argue that zonation is controlled by ambient irradiance. Furthermore, experimentally toppled columns, which experienced immediate and severe changes in irradiance gradients, largely re-established expected patterns of symbiont zonation during a six-month period (Fig. 2e,f). This response shows that the patterns are maintained dynamically.

Symbioses between corals and dinoflagellates are stable mutualisms, with the notable exception of coral bleaching, which involves the loss of symbionts and/or photosynthetic pigments^{3,8}. Bleaching is an ecologically important but poorly understood response to environmental stress^{3,8,9}. Many bleaching events exhibit intra-specific variation distributed within and among habitats in ways

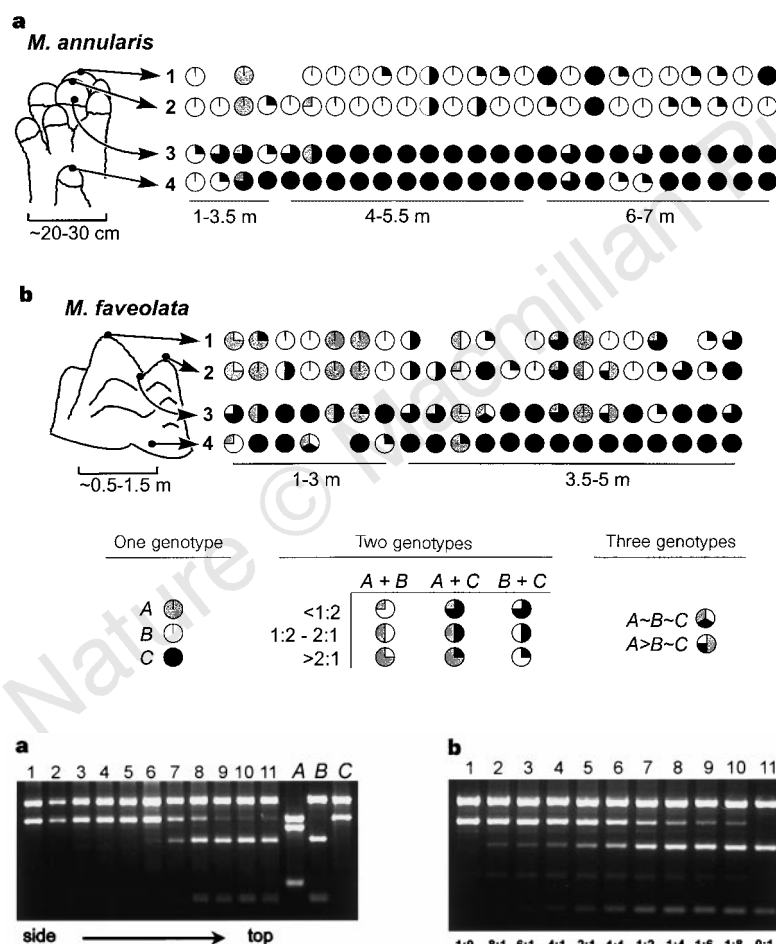


Figure 1 Symbiont communities in *M. annularis* (a) and *M. faveolata* (b). Each symbol represents one core sample that contained *Symbiodinium* A, B, C or mixtures of taxa summarized according to the code shown below. Columns in the data matrices represent individual coral colonies (depth increases from left to right), and rows represent locations of higher (rows 1 and 2) and lower (rows 3 and 4) irradiance, as defined in the diagrams to the left. Samples were collected in January 1995.

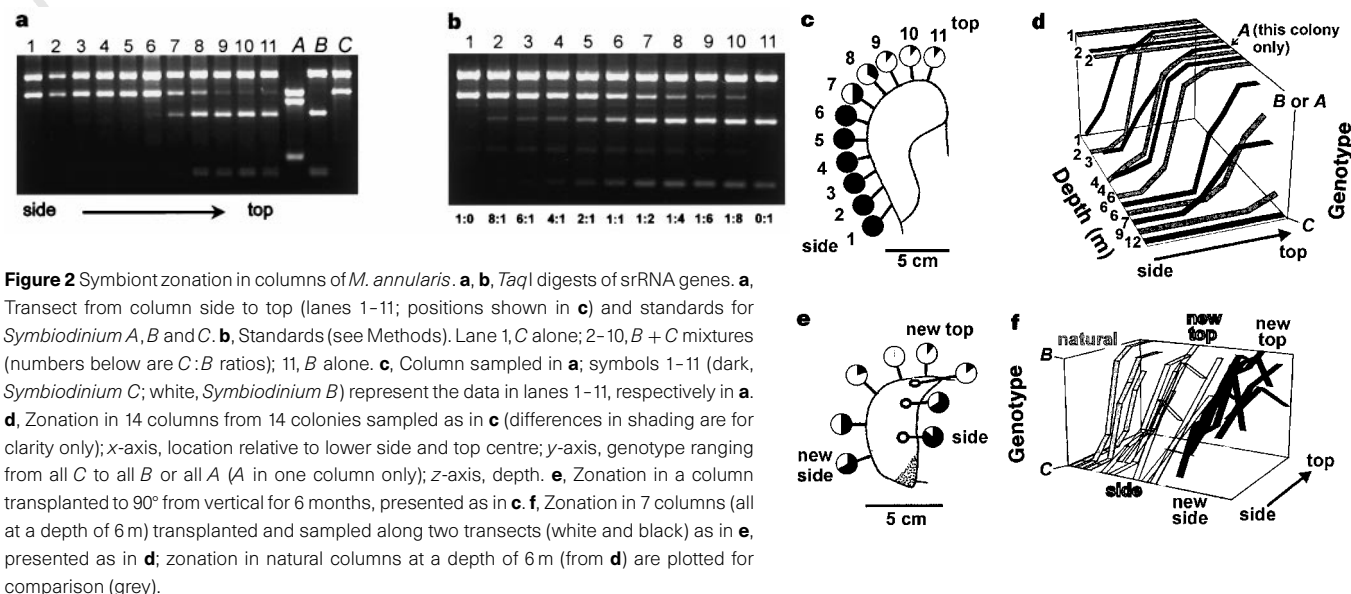


Figure 2 Symbiont zonation in columns of *M. annularis*. a, b, TaqI digests of srRNA genes. a, Transect from column side to top (lanes 1–11; positions shown in c) and standards for *Symbiodinium* A, B and C. b, Standards (see Methods). Lane 1, C alone; 2–10, B + C mixtures (numbers below are C:B ratios); 11, B alone. c, Column sampled in a; symbols 1–11 (dark, *Symbiodinium* C; white, *Symbiodinium* B) represent the data in lanes 1–11, respectively in a. d, Zonation in 14 columns from 14 colonies sampled as in c (differences in shading are for clarity only); x-axis, location relative to lower side and top centre; y-axis, genotype ranging from all C to all B or all A (A in one column only); z-axis, depth. e, Zonation in a column transplanted to 90° from vertical for 6 months, presented as in c. f, Zonation in 7 columns (all at a depth of 6 m) transplanted and sampled along two transects (white and black) as in e, presented as in d; zonation in natural columns at a depth of 6 m (from d) are plotted for comparison (grey).

that are difficult to explain^{9–12}. Because irradiance and temperature act synergistically to induce bleaching^{12–14}, and the symbionts of *M. annularis* and *M. faveolata* exhibit different associations with irradiance (Figs 1 and 2), we hypothesized that symbiont polymorphism underlies this variation.

We observed 'paling' in several colonies of *M. annularis* and *M. faveolata* on 18 September 1995, and bleaching was extensive by the second week in October, both in Panama and elsewhere¹⁵. At our study site, this event was 'typical': like a similar event there in 1983 (ref. 16), it followed a prolonged excursion above the mean summer maximum of temperature (Fig. 3e); it also coincided with atypically high water clarity (data in ref. 17), which implies increased irradiance at depth². We also observed complex^{9,10,18} bleaching patterns in both *M. annularis* and *M. faveolata*. Bleaching was rare or slight at both shallow (<2 m) and deep (>15 m) sites; in between, however, both species displayed a curious pattern, with shallower colonies bleached preferentially in shaded places (Fig. 3a,b) and deeper colonies bleached preferentially in unshaded places (Fig. 3c,d). Among *M. annularis* partitioned as in Fig. 1a (communities 1 and 2 versus 3 and 4) and by depth (above 8 m versus below 8 m), this difference was significant ($n = 76$ colonies, 64 bleached; $P < 0.05$, χ^2 test). Some colonies exhibited a 'ring' of bleaching at the boundary between column top and side (Fig. 3a). *M. faveolata* colonies are not easily partitioned into two distinct irradiance microhabitats, but they clearly showed the same reciprocal pattern (Fig. 3b,d), with a shallower (~6 m) centre. Such observations have previously been hard to explain¹² because the environment is isothermal, and the associations with irradiance and colony morphology are inconsistent.

Symbiont zonation provides a simple hypothesis to explain these bleaching patterns. Bleaching was disproportionately common in what seems to be the upper limit of *Symbiodinium C*'s 'adaptive zone': low-irradiance parts of corals in shallower water, and high-irradiance parts of corals in deeper water. Slight increases in temperature and irradiance might push these symbioses, but not

others, beyond some physiological limit, resulting in bleaching. This hypothesis accounts for our bleaching observations, including areas of slight bleaching^{9,15,19} (see Fig. 3a, b), if *Symbiodinium C* were expelled selectively from mixed symbiont communities.

An analysis of symbionts collected in late October supported this interpretation of events. Post-bleaching samples were obtained <1 cm from many sites sampled the previous January (Fig. 1). All available samples from communities that had previously contained mixtures of *Symbiodinium C* plus either A or B (or both) were identified (Fig. 1) and analysed. We reasoned *a priori* (Fig. 2) that these sites accurately defined the limit of *Symbiodinium C* in corals under non-bleaching conditions. Such mixtures also allow the fates of different symbionts to be compared directly. We also tested archived samples taken at the same time as, and <1 cm away from, the original (pre-bleaching event) samples. In every case, *Symbiodinium* srRNA RFLPs in these replicate, pre-bleaching pairs were equivalent (data not shown), indicating that the small distance between pre- and post-bleaching samples was unlikely to be significant.

As predicted, *Symbiodinium C* had decreased in relative abundance in all 18 communities tested (see Fig. 4a–c). Absolute responses of different symbionts within a mixed community were compared by using estimates of relative abundances (from RFLP data; see Fig. 4a–c) to partition direct counts of symbionts into each genotype (Fig. 4d). Losses of *Symbiodinium C* were typically close to 100%, whereas B underwent a median decrease of 14%, and A more than doubled in 3 of 5 instances. The single sample that contained all three symbionts exhibited these same trends (Fig. 4c, d). Changes in colony chlorophyll contents and subjective assessments of bleaching paralleled changes in symbiont numbers (Fig. 4e). From these data we can tentatively rank the 'fitness' of the different *Symbiodinium* taxa as symbionts under 'bleaching conditions'. The ranking obtained in this manner is: $A > B \gg C$.

Our study provides a fuller understanding of *M. annularis* and *M. faveolata*, which are dominant members of western Atlantic reefs²⁰

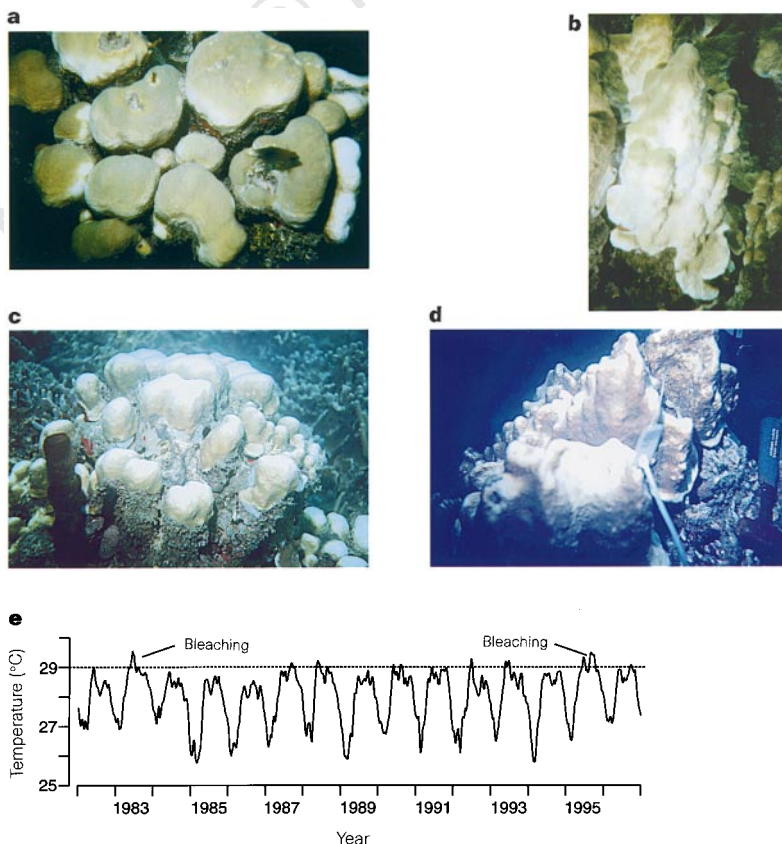


Figure 3 Bleaching in *M. annularis* (a, c) and *M. faveolata* (b, d) at the study site on 28 October 1995 showing 'shallow' (a, b) and 'deep' (c, d) patterns. e, Sea surface temperatures (three-week running means, from satellite data³⁰) at the San Blas Islands, Panama. Temperatures above 29 °C in 1983 and 1995 were associated with coral bleaching¹⁶ (this study). Records from our study site (at 7-m depth) since 1993 (Marine Environmental Sciences Program, Smithsonian Tropical Research Institute) corroborate satellite data.

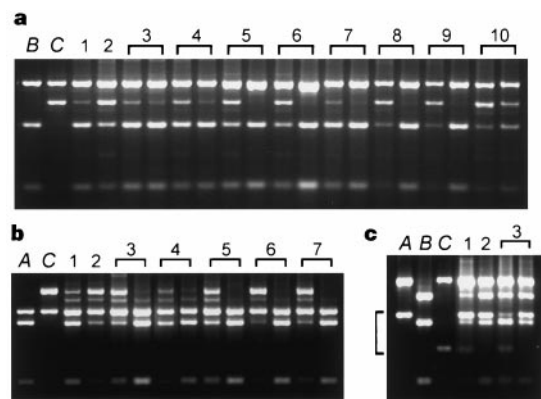
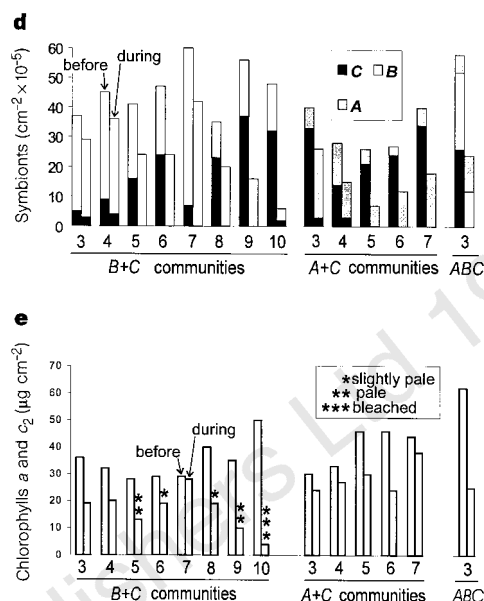


Figure 4 Symbiont communities before (January 1995) and during (October 1995) an episode of coral bleaching. **a-c**, Lanes contain *TaqI* (**a**, **b**) or *DpnII* (**c**) digests of srRNA genes. **a**, Standards for *B*, *C* and *B*:*C* ratios of 8:1 (lane 1) and 1:1 (lane 2); lane pairs compare symbionts before (left) and during (right) bleaching in *M. annularis* (lanes 3–6) and *M. faveolata* (lanes 7–10). **b**, Standards for *A*, *C* and *A*:*C* ratios of 2:1 (lane 1) and 1:8 (lane 2); lane pairs 3 (*M. annularis*) and 4–7 (*M. faveolata*) compare symbionts as in **a**. **c**, Standards for *A*, *B*, *C* and equal amounts of *A*, *B* and *C* (lane 1) and *A* and *B* (lane 2); lane pair 3 compares symbionts in *M.*

and are widely used as model systems in reef biology and geology^{11,13,18,19,21,22}. Each coral 'species' encompasses one animal and dynamic, multi-species communities of symbiotic dinoflagellates. This strongly contradicts the 'one host, one symbiont' view of reef corals¹, in which host taxa alone are adequate units of biodiversity, environmental variability is accommodated largely by physiological acclimatization^{2–4}, and bleaching variability is often not understood¹². We conclude that polymorphic symbiont communities underlie the broad distributions²⁰ and bleaching ecology of these corals. Directed shifts in symbiont populations following extreme environmental change (Figs 2e, f and 4) suggest that similar shifts may also occur over annual cycles of environmental variation¹⁹. For these corals, and for mutualisms in general, the ability to cope with environmental change through changes in symbiont community composition reflects the selective advantage of hosting several distinct symbionts, despite the potential for destabilizing competition among them^{5,6}.

How typical and important are the patterns documented here? *M. annularis* and *M. faveolata* in the Bahamas also host *Symbiodinium* *A*, *B* and *C* (data not presented), and published photographs¹⁸ and descriptions^{9,10} of bleaching elsewhere strongly resemble our own (Fig. 3a–d). With respect to Caribbean corals in general, bleaching is often predominant at intermediate depths⁹. We can attribute this pattern (and its within-colony correlate) in *M. annularis* and *M. faveolata* to symbiont polymorphism and zonation. Moreover, at least three other species of Caribbean corals host (at least) both *Symbiodinium* *A* and *C* (ref. 23; our unpublished data). For other species, which might host multiple but not so distantly related symbionts, refinements of *Symbiodinium* taxonomy would be essential. However, symbiont polymorphism does not exclude the significance of other attributes that are important features of coral biology, such as physiological acclimatization of hosts and symbionts^{2–4} and genetic differences among hosts^{11,14}.

It has been hypothesized that a global warming trend, with increased frequencies of world wide coral bleaching induced by



faveolata, as in **a** and **b**. The vertical bracket identifies bands that identify each symbiont. **d**, Densities of *A* (grey), *B* (white) and *C* (black) before and during bleaching (left and right bars of each pair, respectively) in samples reported in **a** (*B* + *C*, communities 3–10), **b** (*A* + *C*, communities 3–7) and **c** (*ABC*, community 3). **e**, Chlorophyll contents of the samples reported above, presented as in **d**. Samples were scored as 'normal' (not marked) or 'slightly pale', 'pale', or 'bleached' (marked by asterisks) when collected.

increasing temperature or ultraviolet irradiation, could have catastrophic consequences for many living coral reefs^{3,8,21}. Alternatively, coral communities may adjust to climate change by recombining their existing host and symbiont genetic diversities^{24–26}. Our findings supply a precedent for this idea: that one species of coral can flexibly host more than one taxon of *Symbiodinium* to produce symbioses with distinct ecological properties. For example, *M. annularis* and *M. faveolata* might adjust to a warmer Atlantic ocean by hosting more *Symbiodinium* *A* and less *Symbiodinium* *B* and *C*. However, long-term consequences of such replacements would depend on how they affect rates of coral growth and reproduction. □

Methods

Field collections and manipulations. Coral samples were collected at Aguadargana reef in San Blas, Panama²⁷ by coring (1.1 cm² surface area) and freezing immediately in liquid nitrogen (data in Figs 1 and 4). Other colonies (data in Fig. 2) were sampled by removing a defined circular area (~0.12 cm²) of living tissue from freshly collected colonies with an airbrush. In transplant experiments (Fig. 2e, f), columns of *M. annularis* were broken off ~15 cm below the living tissue, turned on their sides, and cemented (at the non-living base) back to the colony at a comparable location; this increased (new top), decreased (new side), or did not change (side) local irradiance. All 28 transplants at a depth of 6 m seemed to be normal after 6 months. Analyses of non-transplanted (control) columns showed that natural zonation patterns were stable over this period (data not shown).

Laboratory analyses. Symbionts and symbiont DNA were isolated from frozen⁷ and from fresh²⁸ samples. Nuclear srRNA genes were amplified using the 'universal' PCR primers ss5 and ss3 (all data in Fig. 1) or a combination of ss5 and the 'Symbiodinium-biased' primer ss3Z (all data in Figs 2 and 4), and analysed with *TaqI* and *DpnII* (data were consistent in every case)⁷. The biased primer (ss3Z) does not discriminate (within this study) against unknown, specific *Symbiodinium* genotypes (discussed in ref. 28), as confirmed by sequencing⁷ and by comparing results from 'universal' and 'biased' amplifications of 30 samples that contained two genotypes (*A* + *C* or *B* + *C*) in various

proportions.

Cloned srRNA genes from *Symbiodinium A*, *B* and *C* were used as standards⁷. They were amplified singly and from defined mixtures of two (Fig. 2b) or three (Fig. 4c) types⁷ to assign field samples to classes of symbiont relative abundance by visual comparison (Fig. 2a, b). To validate this procedure, approximately equal numbers of *A*, *B* and *C* cells, from three natural isolates of each type, were mixed in pairwise combinations and analysed. The results implied that *Symbiodinium B* and *C* yield (on a per-cell basis) equal signals, whereas *A* yields about twice that amount. Standard mixtures of cloned genes were adjusted accordingly.

Symbiont densities and chlorophyll contents (Fig. 4d, e) were determined from haemocytometer counts (8 replicate grids per sample) and spectrophotometrically from methanol extracts²⁹, respectively. These symbionts were isolated quickly (with minimal washing) from frozen samples at 4 °C under dim light. Symbiont genotypes, numbers and chlorophyll contents were obtained from subsamples of each isolate.

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A polymorphism maintained by opposite patterns of parasitism and predation

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Although polymorphism is a widespread phenomenon that has been recognized for nearly two centuries, the basic mechanisms maintaining most polymorphisms in nature are unknown^{1,2}. We present evidence that a polymorphism can be maintained exclusively by balanced selection from two predatory species. For field and laboratory experiments, we used the pea aphid, *Acyrtosiphon pisum*, which occurs as 'green' and 'red' colour morphs, and two species that attack pea aphids, the parasitoid *Aphidius ervi* and the predator *Coccinella septempunctata*. We found that when parasitism rates in the field were high relative to predation rates, the proportion of red morphs increased relative to green morphs, whereas the converse was true when predation rates were high relative to parasitism rates. Detailed laboratory and field studies confirmed that green morphs suffer higher rates of parasitism than red morphs, whereas red morphs are more likely to be preyed on by predators than green morphs are. We present a mathematical model that demonstrates that biased density-dependent parasitism and/or predation on different morphs is sufficient to maintain the colour polymorphism in the population. Our findings support an important role for predation in the maintenance of genetic diversity.

Aphids occur in a range of colour morphs that can differ in growth rates, host range, defensive behaviour, and susceptibility to parasitism^{3–6}. Pea aphids, *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae), in south-central Wisconsin occur as two colour morphs, green and red. The colour morphs remain distinct through the summer months because the aphids reproduce parthenogenetically. Pea aphids experience high levels of parasitism by the wasp *Aphidius ervi* Haliday (Hymenoptera: Aphididae) and heavy predation by several predators, including ladybird beetles, especially *Coccinella septempunctata* L. (Coleoptera: Coccinellidae). *A. ervi* is a 'parasitoid' that attacks aphids by inserting an egg through the aphid's cuticle; the developing wasp larva feeds on and eventually kills the aphid⁷. Both the parasitoid and the predator can have a major impact on pea aphid populations^{8,9} and hence may be important selective agents. Although all three species are introductions to the Nearctic, they have a long evolutionary history in their common Palearctic home range, where both aphid colour morphs also coexist^{10–12}.

In field populations, we found that the relative level of parasitism and predation had a significant effect on aphid colour morph composition. Specifically, the proportion of red morphs increased following relatively high parasitism and decreased following relatively high predation (Fig. 1), implying balancing selection by parasitism and predation. This balanced parasitism/predation hypothesis was supported by our further studies demonstrating directly that parasitism by *A. ervi* is heavier on green morphs, whereas predation by *C. septempunctata* is heavier on red morphs. The parasitism rate on green morphs in the field (53%) was significantly higher than on the red morph (42%) ($P < 0.001$). Significantly higher parasitism rates on a green morph over a red morph have also been reported for the alfalfa aphid, *Macrosiphon creelii* Davis¹³. Another study found higher parasitism on red than on green morphs of *A. pisum*⁴: the difference between this result and

ours may be due to differences in experimental protocol (that is, laboratory rather than field) or differences in the aphid or parasitoid strains used.

The high mobility of ladybird beetles made it impossible for us to measure predation in the field. However, we measured predation rates by *C. septempunctata* on caged plants in a greenhouse, and the predation rate on red morphs (0.91 ± 0.08 aphids eaten per hour) significantly exceeded that on green morphs (0.73 ± 0.06 aphids eaten per hour) ($P < 0.04$). Thus the observed pattern of parasitism and predation supports the hypothesis that the polymorphism is maintained by a balance of these two sources of mortality.

In all previous studies in which predation is a selective force maintaining a polymorphism, susceptibility to predation of some morphs is balanced against some other trait which makes susceptible morphs more competitive in the absence of predation. We found no evidence for this type of balance. Reproductive rates of the two morphs were not significantly different ($P = 0.437$). Thus, a hypothesis that greater parasitism or predation rates on one morph were balanced by greater reproductive rates is not supported. Furthermore, the propensity to drop from a plant when confronted with a foraging predator, an important aphid defensive behaviour, did not differ significantly between the two morphs ($P = 0.226$; see ref. 14 for methods). Although there may be other more subtle differences in the biology of the two colour morphs¹², the evidence implicates balanced parasitism and predation as the factors that maintain the colour polymorphism.

The differential susceptibility of colour morphs suggested that the parasitoid and predator may be using prey colour as a foraging cue, and both *A. ervi* and *C. septempunctata* have been shown to use visual cues to locate prey^{15,16}. To investigate the use of colour cues by

C. septempunctata, we measured predation rates on both morphs in red, green or white containers. In green containers, predation by *C. septempunctata* was higher on the red morph, whereas in red containers predation was higher on the green morph. There was no difference in predation between the morphs in white containers (Table 1). These results indicate that red morphs are more susceptible on green plants because they are more visible.

The explanation for higher *A. ervi* parasitism on the green morph is not as obvious, as the green morph should be relatively more cryptic visually than the red morph on green plants. In an experiment similar to that on *C. septempunctata*, *A. ervi* showed no effect of container colour on relative parasitism rates on the two morphs. However, neither visual nor olfactory discrimination of the aphid morphs by the parasitoids can be completely ruled out by these experiments. In addition, differential encapsulation of parasitoid eggs is a possible mechanism for different parasitism rates on the morphs, which does not involve discrimination by the parasitoid⁴. However, we found no evidence of encapsulation in dissections of over 2,000 aphids containing eggs. Despite the clear evidence for higher parasitism rates on green morphs in the field, the mechanism for this pattern is unknown.

To examine the consequences of morph-biased parasitism and predation, we constructed a simple model of aphid morph–parasitoid–predator population dynamics (Box 1). A central assumption of the model is that one or both of the predatory species will exert density-dependent mortality on the aphids. As pea aphids make up the vast majority of *A. ervi* hosts in agricultural systems, *A. ervi* population dynamics are coupled to those of its hosts, so density dependence can be assumed. Although *C. septempunctata* is a generalist predator with a wide host range, it exhibits aphid-

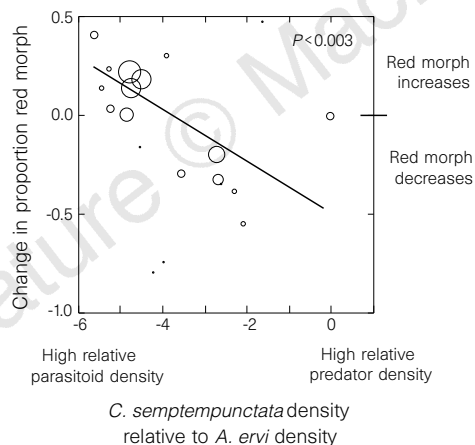


Figure 1 Change in proportion of red morphs in the aphid population between successive field samples versus the relative predation pressure. The sizes of the circles are proportional to weightings based on sample sizes. The regression is statistically significant at the $P < 0.003$ level ($n = 20$).

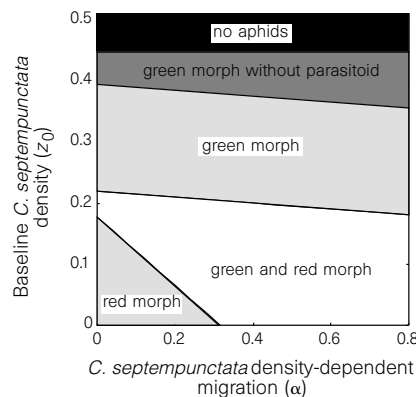


Figure 2 Theoretical conditions leading to coexistence of green and red aphid morphs. Combinations of z_0 and α are shown that satisfy the condition for coexistence of colour morphs. Although details depend on the particular equations and values of the parameters used (Box 1), the qualitative structure of the figure—particularly the existence of a region of morph coexistence—is the same for all biologically plausible models.

Table 1 Numbers of morphs eaten on different backgrounds

Background	Red morphs eaten	Green morphs eaten	<i>P</i>
Green	2.10 ± 0.31	1.45 ± 0.31	0.014
Red	0.80 ± 0.16	1.45 ± 0.26	0.014
White	1.40 ± 0.24	1.55 ± 0.30	0.563

The numbers of green and red pea aphid morphs (mean $\pm$ s.e.) eaten by *C. septempunctata* on different coloured backgrounds are shown.

Table 2 Distribution of *C. septempunctata* relative to aphid density

Source*	df	F-ratio	<i>P</i>
Aphid density	1	5.56	0.025
date	5	3.23	0.019
error	30	0.488	

* The estimate of the slope (α) is 0.60, and the intercept (z_0) is estimated as the average for all dates; $z_0 = 0.11$.

Box 1 General mathematical model

A model to investigate the potential for balanced predation and parasitism to maintain two colour morphs can be derived as follows. Let x_1 and x_2 denote the densities of green and red morphs, and y and $z[x_1, x_2]$ the densities of *A. ervi* and *C. septempunctata*, respectively; density-dependent migration makes $z[x_1, x_2]$ depend on the number of green and red morphs in the field. The population dynamics may be described by

$$\begin{aligned}\frac{dx_1}{dt} &= x_1 f[x_1 + x_2, (1+p)y + bz[x_1, x_2]] \\ \frac{dx_2}{dt} &= x_2 f[x_1 + x_2, y + (1+q)bz[x_1, x_2]] \\ \frac{dy}{dt} &= yg[(1+p)x_1 + x_2 + x_2, y]\end{aligned}$$

where f and g are functions for the per capita population growth rates of aphids and *A. ervi*, respectively. We assume that interactions between aphid morphs are symmetrical, so f depends on the sum $x_1 + x_2$. The higher parasitism rate on the green morph is modelled by augmenting the parasitism rate by a fraction p above that for the red morph, and predation by *C. septempunctata* on the red morph is augmented by a fraction q . Finally, b is the predation rate by *C. septempunctata* relative to the parasitism rate by *A. ervi*.

For any biologically plausible functions f and g , there is always a range of values of p and q that produce coexistence of green and red morphs. For functions $f[x, y]$ and $g[x, y]$, let x and y represent aphid density and combined predator densities. It is biologically reasonable to assume that $df/dx \geq 0$, $df/dy < 0$, $dg/dx \geq 0$, and $dg/dy \leq 0$. Further, let x_1^* and y_1^* denote the aphid and *A. ervi* densities at equilibrium in the absence of the red morph, and define x_2^* and y_2^* similarly when the green morph is absent. Both morphs will coexist whenever $y_1^* > q/pbz[x_1^*]$ and $q/pbz[x_2^*] > y_2^*$. As *A. ervi* has a higher parasitism rate on the green morph, y_1^* will always be greater than y_2^* . Furthermore, the higher parasitism rate on green morphs drives down the morph density, so $x_1^* \leq x_2^*$ and consequently $z[x_1^*] \leq z[x_2^*]$. Therefore, it is always possible to select values of p and q that produce coexistence. (Detailed mathematical proofs will be provided on request.) Note that if *C. septempunctata* did not show aphid-density-dependent migration ($z[y_1^*] = z[y_2^*]$) and *A. ervi* was not a specialist ($y_1^* = y_2^*$), then no coexistence is possible.

The particular equations used for our numerical example (Fig. 2) are

$$\begin{aligned}\frac{dx_1}{dt} &= rx_1 \left(1 - \frac{x_1 + x_2}{K}\right) - a(1+p)x_1y - bx_1z[x_1, x_2] \\ \frac{dx_2}{dt} &= rx_2 \left(1 - \frac{x_1 + x_2}{K}\right) - ax_2y - (1+q)bx_2z[x_1, x_2] \\ \frac{dy}{dt} &= cy((1+p)x_1 + x_2) - dy\end{aligned}$$

which employ logistic aphid population growth, a linear per capita parasitism rate by *A. ervi*, a , and a linear predation rate by *C. septempunctata*, b . We assume that the number of *C. septempunctata* in a field is given by $\sqrt{z[x_1, x_2]} = z_0 + \alpha(x_1 + (1+q)x_2)$. Differential parasitism and predation on the two morphs are included as $(1+p) = (0.53/0.42) = 1.26$ and $(1+q) = (0.91/0.74) = 1.23$ obtained from our experiments. The aphid intrinsic rate of increase, r , is estimated as 0.2 day^{-1} . The parameter K acts as a scaling parameter of aphid density and was arbitrarily set to 1. We assume that per capita parasitism and predation rates are the same, $b = 1$. Other parameter values are $a = 1$, $c = 0.5$ and $d = 0.25$. □

density-dependent migration^{9,17–19}. We confirmed the importance of aphid-density-dependence in our system by showing that there were statistically significantly more *C. septempunctata* found in fields with more aphids (Table 2).

The model shows that the coexistence of the two colour morphs is facilitated by both density-dependent parasitism resulting from the coupling of host and parasitoid population dynamics, and density-dependent migration that leads to greater predator densities in fields with high aphid abundance. Although the density dependence of *A. ervi* that results from its population dynamics being coupled to those of the aphids promotes morph coexistence, even in the absence of *C. septempunctata* density dependence, the potential for coexistence is greatly enhanced if the predator exhibits density dependence as well (Fig. 2). Thus the maintenance of the colour polymorphism is not simply a matter of two predatory species preferentially attacking alternate morphs; it also requires density-dependent mortality from the parasitoid and/or predator.

The discovery that balancing parasitism/predation can maintain a polymorphism indicates that other unexplained polymorphisms may be maintained by balanced density-dependent mortality from predators, parasitoids and/or pathogens, as almost all organisms are killed by a complex of predatory species. Determining an underlying selective mechanism of polymorphism maintenance bears directly on the long-standing debate on whether the bulk of polymorphisms should be classified 'adaptive' or 'neutral'^{20,21}. Our results also have implications for the conservation of biodiversity, given the importance of preserving genetic diversity within insect species in addition to conserving the species themselves^{22–24}. An understanding of the mechanisms that maintain genetic diversity will enable conservation programs to be more effective. □

Methods

Assessment of parasitoid, predator, and aphid densities in the field. We estimated the density of the parasitoid, the predator, and the two aphid morphs by sampling 12 alfalfa fields roughly every 6 days throughout the summer of 1996. In each field, aphid density and colour were recorded for 100 stems in eight locations. Twelve three-minute walking scan samples of parasitized aphids and *C. septempunctata* adults were also made throughout the field. Parasitoid counts were limited to 'mummified' pea aphids which are immobile, skeletonized aphids containing one parasitoid larva or pupa. Sampling dates followed by an alfalfa harvest and fields without aphids were excluded from the analysis.

Impact of parasitoids and predators on relative proportions of colour morphs. We measured the impact of parasitoids and predators on the relative proportions of colour morphs by regressing the change in proportion of red morphs in the aphid population between successive field samples versus the relative predation pressure. The relative predation pressure was measured as $\log[(C7_{t-1} + 1)/(C7_{t-1} + m_t)]$, where $C7_{t-1}$ is the number of *C. septempunctata* observed during 12 three-minute scan samples in a field in week $t - 1$, and m_t is the number of mummies observed in the field during scan samples in week t . We used m_t as a measure of parasitism in week $t - 1$ because mummies form roughly one week after parasitism. The regression of $\Delta r = r_t - r_{t-1} = \text{constant} + \text{slope} \cdot \log[(C7_{t-1} + 1)/(C7_{t-1} + m_t)]$ was done while weighting by the square-root of the total number of aphids counted in either week $t - 1$ or week t , whichever had the fewest aphids. Weighted regression was necessary to correct for higher variance in Δr created when the number of aphids counted per field was small.

***C. septempunctata* density dependence.** We tested for aphid-density-dependent migration by *C. septempunctata* by regressing aphid density (at each sample date) versus *C. septempunctata* density. The regression model was $\sqrt{C7} = z_0 \cdot \text{date} + \alpha \cdot A/\bar{A}$, where $C7$ is the number of *C. septempunctata* observed per field in 12 three-minute scan samples, A is the number of aphids per 800 alfalfa stems, $\bar{A}$ is the mean number of aphids per 800 stems for all fields, and 'date' is a categorical variable for the date of the sample. Only those dates on which *C. septempunctata* were found in two or more fields are included.

Assessing parasitism rate. To estimate parasitism rates, we sampled five

alfalfa fields on five dates in May, June and August 1996, and dissected a total of 643 aphids for larval *A. ervi*. Data were analysed with χ^2 test, blocked by field and date.

Susceptibility of colour morphs to *C. septempunctata* on plants. We measured the susceptibility of the two aphid colour morphs to predation by allowing a single adult *C. septempunctata* to forage on caged alfalfa plants with 15 adults of each morph for 4 h; the experiment was replicated 27 times. After removing the predator from each plant, the number of remaining aphids of each morph was recorded and subtracted from 15 to determine the number consumed. The data were analysed with a 2-tailed, paired *t*-test.

Use of colour cues by *C. septempunctata*. We investigated the use of colour cues by *C. septempunctata* by allowing single adult beetles to forage for aphids of both colour morphs in red, green and white containers. To maximize our ability to detect differences in predation rates between morphs, we designed the experiment as a split-plot, with background as the whole plot and aphid colour morph as the split plots. Each arena had a single background colour and 5 similar-sized (third or fourth instar) aphids of each colour morph. Predators foraged for 30 min, and at the end of the experiment the number of remaining aphids of each colour morph was recorded and subtracted from 5 to determine the number consumed. All predators foraged actively and consumed at least one aphid. Each treatment was replicated 20 times. Data were analysed as a split-plot analysis of variance.

Comparison of reproductive rate of the colour morphs. The reproductive rates of the aphid colour morphs were compared by pairing 10 of each morph on individual caged alfalfa plants and allowing them to reproduce for 14 days. At the end of the experiment, all aphids of each morph were recorded. The experiment was started with 14 plants, but 2 plants were severely wilted after two weeks so these were excluded from the analysis. Data were analysed with a 2-tailed paired *t*-test.

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Multistability of cognitive maps in the hippocampus of old rats

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Hippocampal neurons provide a population code for location¹. In young rats, environments are reliably 'mapped' by groups of neurons that have firing locations ('place fields'²) that can be stable for several months³. Old animals exhibit deficits in spatial memory, raising the question of whether the quality or stability of their hippocampal 'cognitive maps'⁴ is altered. By recording from large groups of neurons, we observed the hippocampal spatial code to be multistable. In young rats, the place field maps were reliable both within and between episodes in a familiar environment. In old rats, place field maps were accurate and stable during an episode, but frequently exhibited complete rearrangements between episodes. In a spatial memory task, both young and old rats exhibited bimodal performance, consistent with map multistability early in training. However, the performance of young rats became almost unimodal with further training, whereas that of old rats remained markedly bimodal. The multistability of the hippocampal map provides an insight into the dynamics of neural coding in high-level cortical structures and their changes during ageing, and may provide an explanation for the frequent failure of place recognition in elderly humans.

Spatial memory is deficient in healthy elderly humans⁵, non-human primates⁶ and rodents⁷. The hippocampus is important for spatial learning⁴, and exhibits complex, region-selective changes in its anatomical connectivity, synaptic physiology and neurochemical organization during ageing^{8–10}, including a decline in its ability to maintain synaptic long-term potentiation (LTP)^{7–11}. Paradoxically, however, there are only small changes in the spatial information conveyed by hippocampal pyramidal neurons in aged animals^{12–14}.

How is this apparent preservation of spatial signalling, despite substantial alterations in cellular physiology and behaviour, to be explained? Place fields can appear in complete darkness during the first trial in an environment and remain unchanged in subsequent illumination¹⁵, suggesting that place-field interrelationships can be prespecified in the synaptic matrix¹⁶, rather than learned or imposed by external stimuli, as earlier theories had suggested^{17–20}. Current evidence indicates that self-motion signals, rather than relationships among landmarks *per se*, are used to specify relative position on a place-field map^{4,16,21}. However, landmark information seems to become bound to map locations through associative learning, and can subsequently provide position fixes that correct for the inevitable drift error in such a self-motion-based navigational system^{21,22}. Associative binding of landmark information to preconfigured maps would also enable the recall of a consistent map for a previously visited environment¹⁶. If place-field maps depend primarily on pre-existing weights within the network but landmark binding depends on LTP of external inputs, then the LTP deficit that occurs in old rats^{7,10,11} might result in the failure to select a consistent map for a given environment, even though the place fields themselves would appear relatively normal.

Multiple CA1 pyramidal cells were monitored simultaneously (number of cells per session (mean $\pm$ s.e.m.): young, 34 ± 13 ; old, 37 ± 16) during the course of two consecutive episodes of running

on an elevated track 'maze' that were separated by ~ 25 min to 1 h, and also during sleep periods before and after this behaviour (see Methods). Only those cells that exhibited spike amplitude stability were included. The between-episode correlations in spike amplitudes were high and identical in the two age groups (young, $r = 0.959$; old, $r = 0.959$). The spike amplitudes and firing rates during the maze episodes were: old, $144 \pm 6 \mu\text{V}$, $0.91 \pm 0.08 \text{ Hz}$; young, $143 \pm 5 \mu\text{V}$, $0.97 \pm 0.10 \text{ Hz}$.

To determine the consistency of place fields, smoothed firing-rate maps²³ (bin size, $1.7 \times 1.7 \text{ cm}$) were constructed for each cell for the first and second maze episodes, and the spatial correlations of these distributions were computed. These correlation coefficients were then averaged across all simultaneously recorded cells to provide an 'ensemble' estimate of the map consistency between episodes. There was a significant difference in mean correlations over trials between age groups (Figs 1 and 2). In young rats, the place-field maps were highly correlated between the first and second maze episodes. In about 70% of sessions, the ensemble correlations

for old rats were also highly correlated; in the remaining sessions, however, there was complete rearrangement of the place fields. The distribution of correlation scores was unimodal in young rats (Fig. 2b) and bimodal in old rats (Fig. 2a), indicating that the rearrangement reflects an all-or-none process ($\chi^2 = 8.69$, $P < 0.03$). Within each maze episode, however, the place fields of old rats were as stable as those of young rats (Fig. 2c,d). This was determined by dividing each of the two maze episodes into halves, and computing the within-episode correlations. These were uniformly high in both age groups (r_{young} , 0.79 ± 0.05 ; r_{old} , 0.77 ± 0.08 ; $\chi^2 = 3.56$, $P > 0.32$). This within-episode stability implies that the unreliability in the old rats is in the selection of the correct cognitive map for a given environment, not in maintaining the map once it is selected.

The old rats sometimes succeed in retrieving the correct map but sometimes do not, suggesting that there may be a bimodality in their performance on spatial tasks. Data with which to explore this question were available from 98 young and 93 old rats (including

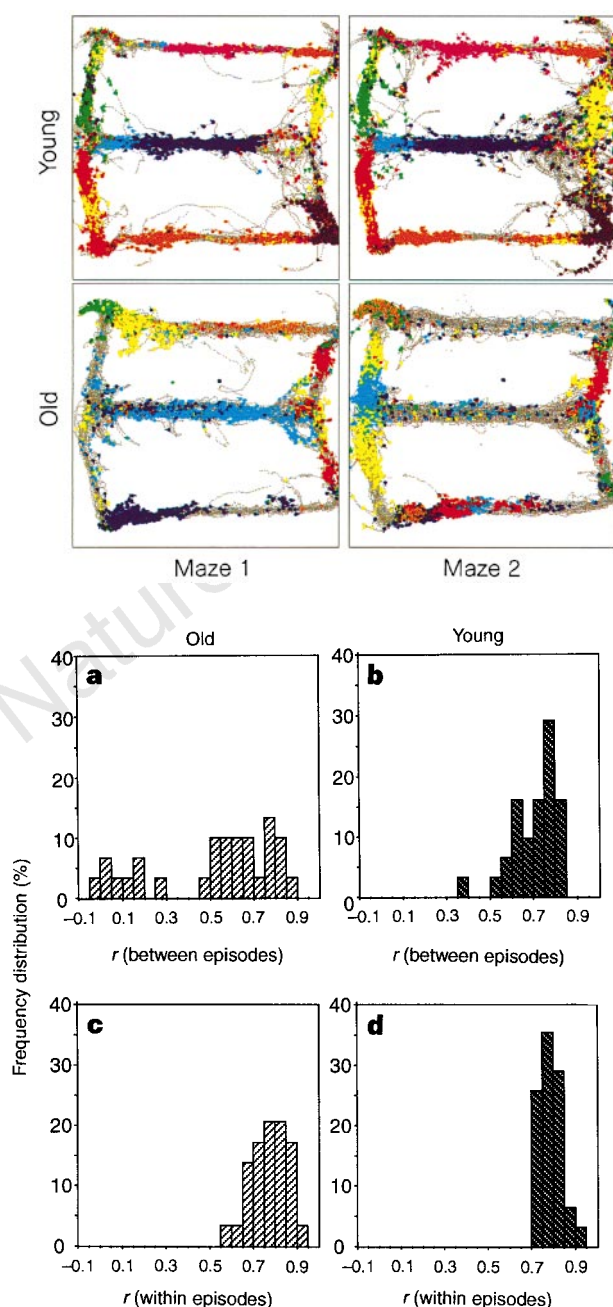


Figure 1 Place-field distributions from one young and one old rat recorded on two consecutive episodes of running on a rectangular figure-8 maze. Between episodes (Maze 1, Maze 2), the rats were removed from the room. The locations at which spikes were emitted are represented as coloured dots, with a different colour for each neuron. The colour scheme is consistent between episodes for each rat. Only a representative subset of the simultaneously recorded cells is illustrated. The grey lines indicate the trajectories of the rats. The spatial clustering of spikes from a given cell defines its place field. The distribution of place fields is referred to as a place-field map. Overlapping sets of place cells are known to participate in different mappings in different environments, but the relationships among place fields in two maps are essentially uncorrelated³⁰. Place-field maps of young animals were typically highly correlated between the two maze episodes on a given day. In old rats the maps were often uncorrelated, even though the abundant visual, auditory, tactile and olfactory cues in the environment were not altered in any way.

Figure 2 a, b. Frequency distributions of average place-field correlations between maze episodes in the (r (between episodes)) for all recording sessions in all rats. Each observation represents a single recording session. For young animals the distribution was unimodal, reflecting a high degree of consistency of the place-field map between episodes. In old animals the distribution was bimodal, with one peak ($\sim 70\%$ of trials) near that of the young animals, and another peak ($\sim 30\%$ of trials) near zero. Taking r (between episodes) = 0.5 as an arbitrary cut-off, the distribution of remappings within the two age groups was as follows (the numerator and denominator indicate the number of remappings and recording sessions, respectively, with the value for old rats followed by that for young rats: pair 1, 1/2, 0/3; pair 2, 3/4, 0/5; pair 3, 3/6, 0/6; pair 4, 1/6, 0/6; pair 5, 1/6, 0/6; and pair 6, 1/6, 1/6. The mean between-episode correlations for each rat were as follows, with the value for old rats followed by that for young rats: pair 1, 0.36, 0.70; pair 2, 0.18, 0.65; pair 3, 0.44, 0.70; pair 4, 0.62, 0.75; pair 5, 0.61, 0.73; pair 6, 0.69, 0.70. The age differences in mean correlation were statistically significant ($F_{1,10} = 7.67$, $P < 0.02$). **c, d.** Within episodes, place-field distributions were highly correlated in both age groups. Thus, without removal from the environment, place fields in old rats were just as stable as in young rats.

the 12 rats in the present study), which were subjects in different experiments, but which were all pretested on the same apparatus using identical procedures (Fig. 3a). Both groups initially exhibited a pronounced bimodality in their performance score distributions (Fig. 3c,e), sometimes exhibiting short swim paths and sometimes long ones. By the last day of training, however, the young rats exhibited only a small proportion of long swim paths, whereas the old rats, although showing some improvement, exhibited about equal proportions of short and long paths. If this bimodality resulted from variation between animals, rather than variation within animals across trials, then the distribution of mean performances within the population of old rats should also be bimodal. The mean path lengths for individual old rats were therefore averaged over the six trials on the last day of testing, when the group behaviour was nearing asymptote. The distributions of mean within-animal performance for old rats was broad, but exhibited no evidence of bimodality (Fig. 3b). Thus old rats as a group continued to exhibit strong bimodality on the last day of training. Performance was also strongly bimodal in young animals earlier in training,

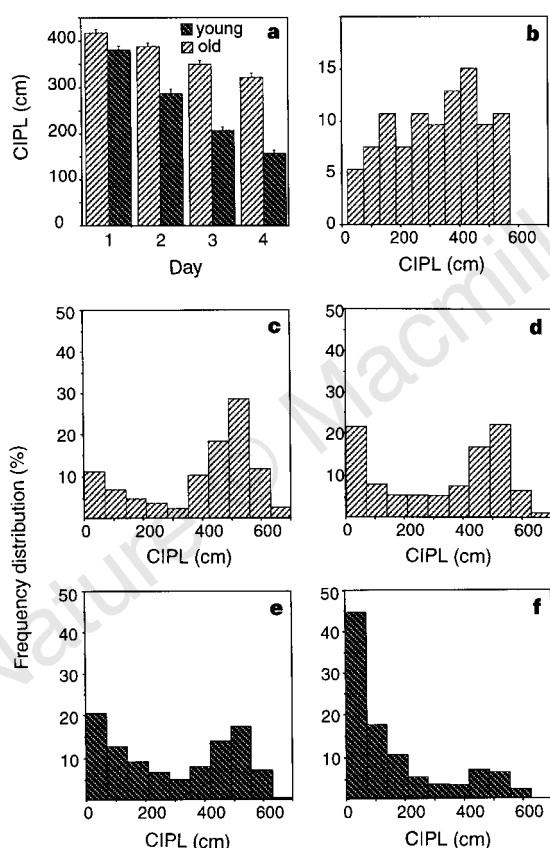


Figure 3 a, Mean performance scores (CIPL; see Methods) versus trial number for 98 young and 93 old rats on the Morris water task. Old rats were significantly impaired at finding the hidden platform in relation to external spatial landmarks. b, Frequency distribution of mean performance scores for old rats during the last six trials (day 4), when performance was nearing asymptote. Although mean performance was variable across rats, there is no evidence that the population of old rats segregates into distinct groups. The bimodal performance illustrated in c-f is thus due to variation within rats, not between rats. c-f, Frequency distributions of performance on individual trials were strongly bimodal in both young and old rats early in training (day 2). By day 4 the performance of young rats was almost unimodal, whereas old rats exhibited about equal proportions of short and long swim paths. The bimodality in path length was not an artefact of the 60-s trial limit used. Removal of the relatively few time-out trials did not appreciably affect the distribution shapes. c, Old rats, day 2; d, old rats, day 4; e, young rats, day 2; f, young rats, day 4.

suggesting that young rats with less experience may exhibit a higher incidence of place-field rearrangements. In a preliminary study¹⁶, a higher incidence was observed in young rats with less total experience in the recording apparatus than in the present study; however, the studies are not quantitatively comparable because of other important procedural differences.

These findings can be understood within a theoretical framework¹⁶ in which the hippocampus contains, within its synaptic matrix, a set of predetermined distributions of place fields ready to be used as spatial coordinate systems for different environments, but not initially bound to particular landmarks or events. The number of available coordinate systems is potentially quite large (about 0.04 times the number of neurons²⁴). During initial exploration, a coordinate system may be selected, and external stimuli may become associated with coordinates through hebbian synaptic strengthening (such as LTP). This could provide a mechanism both for establishing spatial relationships among arbitrary features and events in an environment, and for selecting the correct map upon entry into a familiar environment. According to this theory, disruption of LTP during ageing^{7,10,11} would result in failures of both functions, even though the place cell activity itself would seem normal.

When different maps are selected, is the selection random or restricted to only a few maps for the same environment? The latter case might lead to slower learning, but ultimately would result in the same asymptotic performance. At present, the data are insufficient to allow a definitive answer to this question, although several studies suggest that the asymptotic performances of young and old rats on spatial tasks do not converge²⁵. To the extent that animals improve their performance, one or a small number of maps gradually wins out, as seems to be the case in young rats.

One alternative to the defective-LTP hypothesis is that the quality of sensory information reaching the hippocampus is insufficient for accurate map retrieval. Old rats exhibit visual impairment²⁶, as well as changes in other sensory modalities, although the old rats in this and other studies were relatively normal on visual association tasks, despite being impaired on spatial tasks. Moreover, the environment used was highly structured and rich in spatial cues of all sensory modalities, and the sensory information received by the old rats was sufficient to enable retrieval of the correct map on most trials and to maintain that map once selected. Finally, both young and old rats exhibit bistable behaviour at some point in training, indicating that explanations invoking learning, rather than sensory impairments, are the more plausible.

In summary, old rats fail to retrieve the same map for a given environment more often than do young rats with equivalent experience. This effect may explain why, in an earlier study²⁷ in which the rats were removed from the apparatus between trials, place fields of old rats were less reliable over trials, and also less spatially specific on average across trials. In the present study, the place fields of old rats were at least as reliable and specific as those of young rats, as long as they remained in the environment. Both young and old rats also exhibited bimodal performance in a place-finding task early in training, but the incidence of 'lost' behaviour declined more rapidly with experience in young than in old rats. Whether the behavioural and neurophysiological phenomena are linked remains to be determined. Nevertheless, the data suggest that old animals have intact frameworks for representing spatial relationships, but cannot recall them in a consistent manner, possibly because of a problem in binding information about the external world to locations in these frameworks. This deficit in the ability to recall correctly the spatial context²⁸ in which an event occurred could contribute to the general decline in episodic memory observed during old age. □

Methods

Behavioural testing. Six pairs of one young (12 months old) and one old (28

months old) F-344 rats were first tested on the Morris swim task (six trials per day for four days), which requires that the rat learns the location of a submerged escape platform in a pool of water, on the basis of external landmarks. The performance measure, corrected integrated path length (CIPL)²⁵, is the sum of the distances from the target minus the shortest possible sum if the rat had swum directly to the platform at its mean speed. As reported previously²⁵, the old rats were impaired on this task (mean CIPL $\pm$ s.e.m. on day 4: young, 1.81 ± 0.61 m; old, 2.76 ± 0.39 m; $F_{1,10} = 4.99$, $P < 0.05$), but they were unimpaired when the platform was not submerged and a distinct visual cue was suspended above it (on final trial: young, 0.15 ± 0.07 m; old, 0.16 ± 0.10 m; $F_{1,10} = 0.90$, $P > 0.36$). Thus the old rats were deficient in spatial memory, a characteristic of hippocampal dysfunction, but exhibited normal visual association memory, which does not depend on an intact hippocampus²⁹.

Neurophysiological recording. The rats subsequently underwent the surgical implantation of a micromanipulator array that carried 12 tetrode recording probes, for parallel recording of groups of hippocampal neurons^{1,21}. The rats were trained using a food reward to traverse a track 6 cm wide in the shape of a rectangular figure 8 (Fig. 1a), located in a moderately illuminated room with prominent visual landmarks and numerous tactile, auditory and olfactory spatial cues. Initially, the rats were confined to the north half of the apparatus by a removable partition that prevented access to or view of the other portion. The rats thus ran repeatedly (15–20 laps per episode) around a rectangular course. Three different manipulations were carried out to investigate the consistency of place-field maps on repeated visits to a fixed environment. The first was to allow the rats to spend ~25 min exploring the unfamiliar (south) portion of the track before returning to the familiar portion. This was carried out twice for each rat. In subsequent manipulations, rats were removed from the recording room for 1 h. In half of these sessions, they were returned to their home room; in the other half, they were allowed free exploration in each of six different rooms, for 10 min each, before returning to the recording apparatus. For five young and four old rats, the recording apparatus for the latter manipulations consisted of the north half of the figure 8. For two old rats and one young rat, the full apparatus (with no partition) was used as the test environment, instead of just the north half. (The details of the behavioural experience sequences are available as Supplementary Information.)

Each recording session thus consisted of 5 phases. Sleep 1, a period of ~30 min as the rat sat quietly and/or slept in a small 'nest'; Maze 1, in which the rat made multiple traversals of the track, always running in the same direction; Treatment, in which the rat was either transferred to the novel portion of the track or was removed from the room; Maze 2, in which the rat was returned to the track in its Maze 1 configuration; and Sleep 2, in which the rat was returned to the 'nest'. For four young–old rat pairs, data were available for two sessions in each of the three recording conditions. For technical and logistical reasons, the number of usable data sets was reduced for two rat pairs (Fig. 2).

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Supplementary information is available in Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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cAMP-induced switching in turning direction of nerve growth cones

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Development of the nervous system depends on the correct pathfinding and target recognition by the growing tip of an axon, the growth cone^{1–3}. Diffusible or substrate-bound molecules present in the environment may serve as either attractants or repellents to influence the direction of growth-cone extension^{4–11}. Here we report that differences in cyclic-AMP-dependent activity in a neuron may result in opposite turning of the growth cone in response to the same guidance cue. A gradient of brain-derived neurotrophic factor normally triggers an attractive turning response of the growth cone of *Xenopus* spinal neurons in culture, but the same gradient induces repulsive turning of these growth cones in the presence of a competitive analogue of cAMP or of a specific inhibitor of protein kinase A. This cAMP-dependent switch of the turning response was also found for turning induced by acetylcholine, but not for the turning induced by neurotrophin-3 (NT-3). Thus, in the presence of other factors that modulate neuronal cAMP-dependent activity, the same guidance cue may trigger opposite turning behaviours of the growth cone during its pathfinding in the nervous system.

Isolated spinal neurons in 14–20 h *Xenopus* cultures were used for these experiments. A microscopic gradient of brain-derived neurotrophic factor (BDNF) was created near the growth cone by pulsatile application of picoliter of BDNF-containing saline with a micropipette^{12,13}. The tip of the micropipette was positioned

100 μm from the centre of the growth cone and at a 45° angle with respect to the direction of neurite extension. The direction and total length of neurite extension were measured after 1 hour's growth in the presence of the gradient. The attractive turning response was revealed by a large percentage of growth cones turning towards the source of BDNF (Fig. 1a,b). Superimposed traces of the trajectory of growth cone extension for a population of neurons (Fig. 1c) and the scatter plot of the final position and total extension of growth cones

(Fig. 1d) showed significant attractive turning of the growth cone in the BDNF gradient ($50 \mu\text{g ml}^{-1}$ in the pipette). No significant turning response was observed when the pipette solution contained either a low concentration ($0.5 \mu\text{g ml}^{-1}$) of or no BDNF (Table 1). The attractive response to BDNF was apparently mediated by tyrosine-kinase receptors, because the effect was abolished by an inhibitor of tyrosine kinase K252a (200 nM; ref. 14). Table 1 summarizes the average turning angle and the

Table 1 Response of *Xenopus* nerve growth cones in chemical gradients

Chemicals in the pipette	Chemicals added in the bath†	[Ca ²⁺] ₀ (mM)	Turning angle (degrees)‡	Net neurite extension (μm)‡	Turning responses (%)§			Number of cells examined
					+	0	–	
None	None	1	1.4 ± 2.3	16.7 ± 2.5	18	65	18	17
None	Rp-cAMPS	1	2.2 ± 3.8	11.6 ± 1.5	30	50	20	10
BDNF ($50 \mu\text{g ml}^{-1}$)	None	1	$14.3 \pm 3.0^{**}$	19.6 ± 1.4	74	17	9	35
	K252a	1	-1.0 ± 2.9	18.1 ± 2.4	25	42	33	12
	None	0.001	1.2 ± 4.5	$24.5 \pm 4.5^*$	24	47	29	17
	Rp-cAMPS	1	$-14.6 \pm 3.7^{**}$	17.2 ± 2.2	6	24	70	17
	Rp-cAMPS	0.001	-2.4 ± 6.4	$19.2 \pm 1.6^{**}$	18	55	27	11
	Sp-cAMPS	1	$20.8 \pm 5.2^{**}$	16.1 ± 1.4	91	9	0	11
	KT5720	1	$-18.4 \pm 5.2^{**}$	22.5 ± 3.3	10	10	80	10
	ACPD	1	$-13.6 \pm 2.9^{**}$	16.6 ± 2.3	0	20	80	10
BDNF ($0.5 \mu\text{g ml}^{-1}$)	None	1	-1.8 ± 4.3	16.1 ± 1.8	27	45	27	11
	Forsklin	1	$13.3 \pm 3.6^{**}$	23.2 ± 3.2	80	20	0	10
	1,9-dideoxy-forsklin	1	-0.1 ± 3.4	18.3 ± 2.3	20	60	20	10
Forsklin (5 mM)	None	1	$21.7 \pm 4.2^{**}$	14.2 ± 1.6	80	20	0	10
	Rp-cAMPS	1	-2.3 ± 4.1	13.7 ± 2.4	18	64	18	11
	None	0.001	$18.0 \pm 4.7^{**}$	$36.5 \pm 4.5^{**}$	80	20	0	10
1,9-Dideoxy-forsklin (5 mM)	None	1	2.5 ± 3.0	15.9 ± 2.9	30	50	20	10
NT-3 ($50 \mu\text{g ml}^{-1}$)	None	1	$9.4 \pm 3.3^*$	16.0 ± 2.3	67	27	7	15
	None	0.001	$14.6 \pm 5.8^*$	$27.6 \pm 4.2^*$	73	13	13	15
	Rp-cAMPS	1	$10.6 \pm 4.4^*$	15.3 ± 4.7	60	33	7	15
ACh (100 mM)	None	1	$13.5 \pm 3.8^{**}$	14.5 ± 1.7	80	7	13	15
	Rp-cAMPS	1	$-9.6 \pm 2.9^*$	14.7 ± 1.8	7	33	60	15

† K252a, 200 nM; Rp-cAMPS (adenosine 3', 5'-cyclic phosphorothiolate-Rp), 20 μM ; Sp-cAMPS (adenosine 3', 5'-cyclic phosphorothiolate-Sp), 20 μM ; KT5720, 200 nM; ACPD ((1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid), 50 μM ; forsklin (6 β -[β' -(piperidino)propyl]-forsklin), 5 μM ; 1,9-dideoxy-forsklin, 5 μM . The drugs were added to the bath solution before the start of the chemical gradient and were present throughout the experiments.

‡ Values represent mean $\pm$ s.e.m. Values marked with * ($P < 0.05$) and ** ($P < 0.01$) were significantly different from the control (first two rows) using non-parametric Kruskal-Wallis test. § Growth cone turning responses were scored as follows: +, percentage of cells showing positive turning towards the source of the chemical (turning angle $> 5^\circ$); cells showing no turning ($|\text{turning angle}| \leq 5^\circ$); –, cells turning away (turning angle $< -5^\circ$).

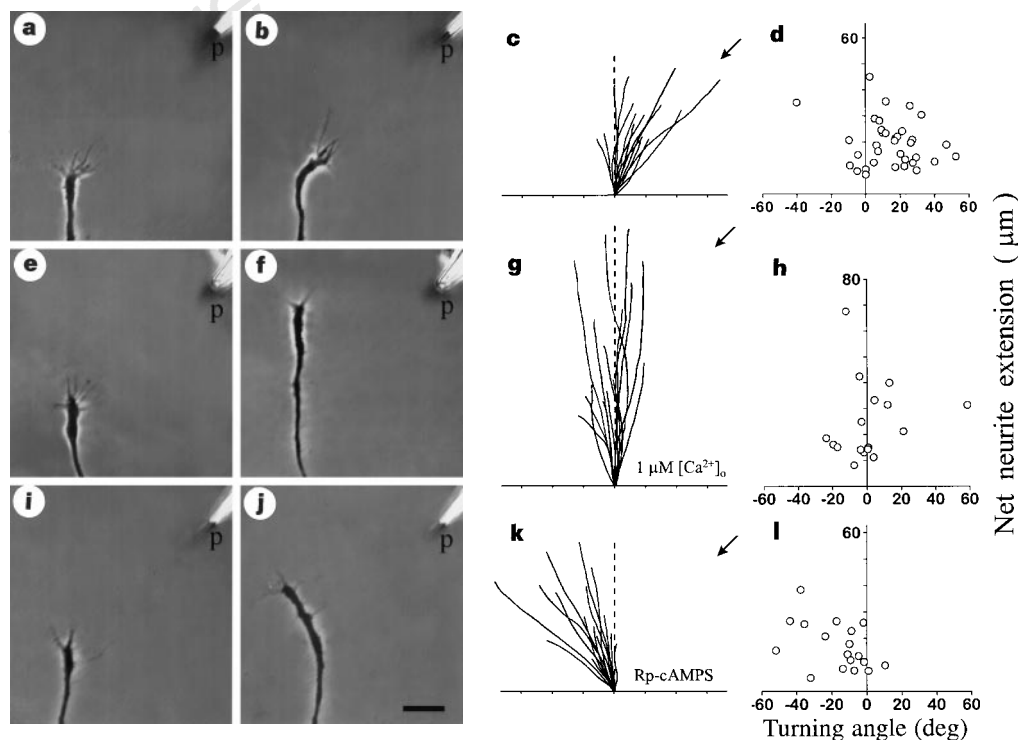


Figure 1 Attractive and repulsive turning of *Xenopus* growth cones induced by BDNF. A gradient of BDNF was applied in normal saline (a–d), in low-Ca²⁺ (1 μM) saline (e–h) and in saline containing 20 μM Rp-cAMPS (i–l). On the left are images of the growth cone at the onset (a, e, i) and at the end of the 1-h period (b, f, j). p: Tip of BDNF-containing pipette ($50 \mu\text{g ml}^{-1}$ in the pipette). Scale bar, 20 μm . On the right are summary plots. Superimposed traces (c, g, k) depict the trajectory of neurite extension during the 1-h period for a sample population of 15 neurons. The origin is the centre of the growth cone at the onset, and the original direction of growth was vertical. Arrows indicate the direction of the gradient. Tick marks, 5 μm . Scatter plots (d, h, l) depict all data collected for each condition. Each point depicts the final angular position of a growth cone (abscissa) and its total neurite extension (ordinate) during the 1-h period.

percentage distribution of growth cones growing towards and away from the pipette.

To investigate the transduction mechanism underlying the turning response, we examined the roles of Ca^{2+} and cAMP, which both affect growth-cone behaviour^{15,16}. First, extracellular Ca^{2+} ($[\text{Ca}^{2+}]_0$) was reduced from the normal level of 1 mM to 1 μM . Consistent with previous findings^{13,17}, this reduction in $[\text{Ca}^{2+}]_0$ led to an increased rate of neurite extension in these neurons. The attractive response was abolished (Fig. 1e–h and Table 1), however, indicating that an influx of Ca^{2+} may mediate the turning response induced by BDNF. The requirement for Ca^{2+} is similar for the potentiation of acetylcholine (ACh) release by BDNF at neuromuscular junctions in these cultures¹⁸. Second, the role of cAMP was examined: when a non-hydrolysable analogue competitor of cAMP, Rp-cAMPS¹⁹, was added to the saline (at 20 μM), nearly all growth cones exhibited repulsive turning in the same BDNF gradient (Fig. 1i–l; Table 1). Bath addition of Rp-cAMPS (20 μM) in the absence of BDNF in the pipette solution had no effect on the direction of growth-cone extension (Table 1). Rp-cAMPS treatment apparently altered the action of endogenous cAMP in these neurons, because the same treatment also abolished the attractive turning response of these

growth cones induced by a gradient of forskolin¹², a drug that activates adenylyl cyclase and endogenous production of cAMP (Table 1). Addition of a specific inhibitor of protein kinase A (PKA), KT5720 (200 nM; ref. 14), to the bath also led to a repulsive turning in the BDNF gradient (Table 1), suggesting that the effect of Rp-cAMPS is probably due to its inhibition of PKA in these neurons¹⁹. Furthermore, bath application of Sp-cAMPS (20 μM ; ref. 19), a cAMP analogue that activates PKA, resulted in a slight enhancement of the BDNF-induced attractive response (Fig. 2a; Table 1). Involvement of cAMP-dependent processes in BDNF-induced turning was also indicated by the following results. In the presence of 50 μM ACPD, a specific metabotropic glutamate receptor agonist that reduces the neuronal cAMP level²⁰, a BDNF gradient triggered clear repulsive responses (Fig. 2b; Table 1). A gradient of BDNF produced with a pipette containing 0.5 $\mu\text{g ml}^{-1}$ BDNF, which was normally ineffective in attracting the growth cone, became highly attractive when forskolin was added to the bath (Fig. 2c,d; Table 1), whereas an inactive analogue, 1,9-dideoxy-forskolin, had no effect (Table 1). Finally, reduction of $[\text{Ca}^{2+}]_0$ to 1 μM completely abolished repulsive turning in the presence of Rp-cAMPS (Table 1), suggesting that both attractive and repulsive

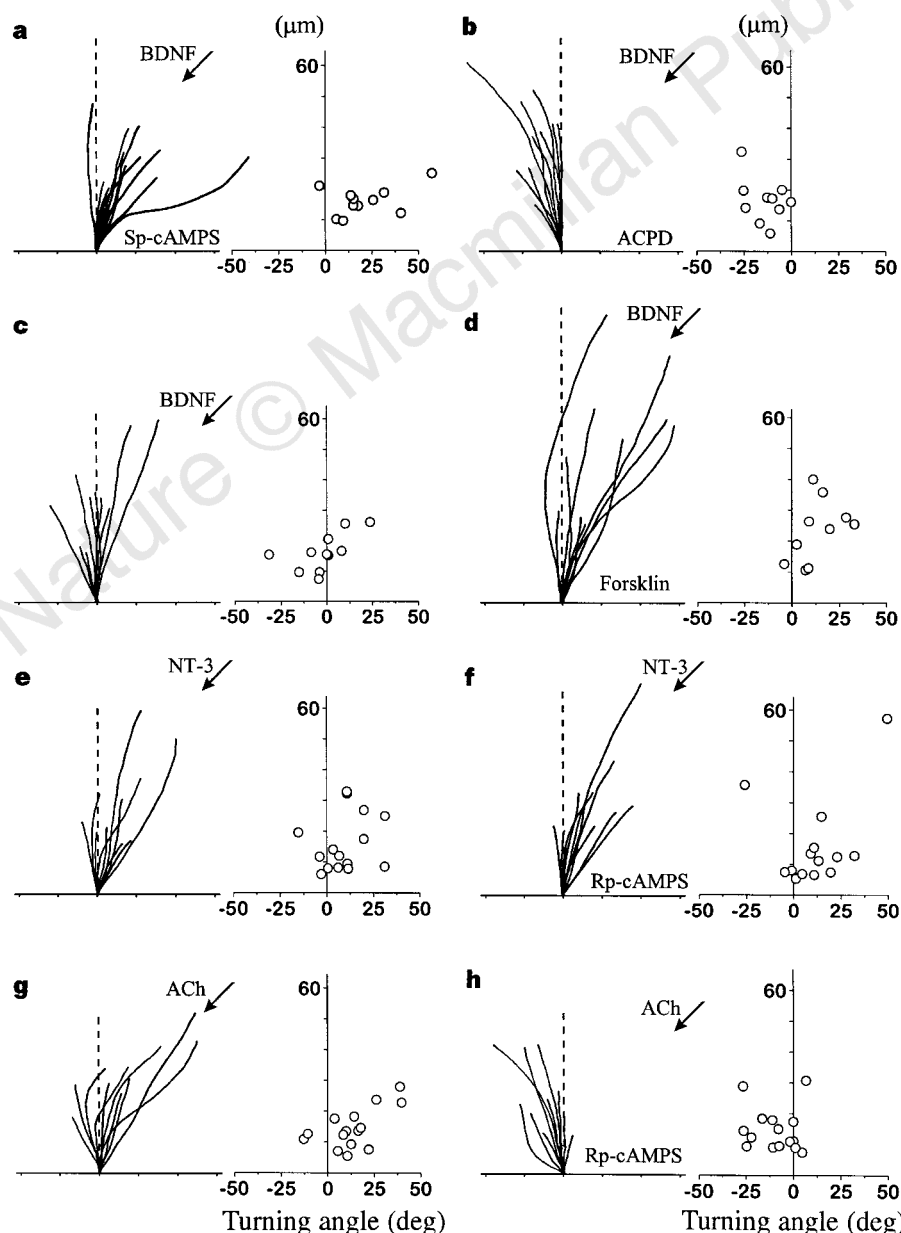


Figure 2 Growth cone turning under a variety of conditions. **a, b**, Turning responses induced by a BDNF gradient (50 $\mu\text{g ml}^{-1}$ in the pipette) in a medium containing Sp-cAMPS (20 μM ; **a**) and ACPD (50 μM ; **b**). **c, d**, Turning responses induced by a gradient of BDNF (0.5 $\mu\text{g ml}^{-1}$ in the pipette) in normal medium (**c**) and a medium containing 5 μM forskolin (**d**). **e–h**, Turning responses in the presence of a gradient of NT-3 and of ACh (50 $\mu\text{g ml}^{-1}$ and 100 mM in the pipette), respectively, in normal medium (**e, g**) and in a medium containing Rp-cAMPS (20 μM ; **f, h**).

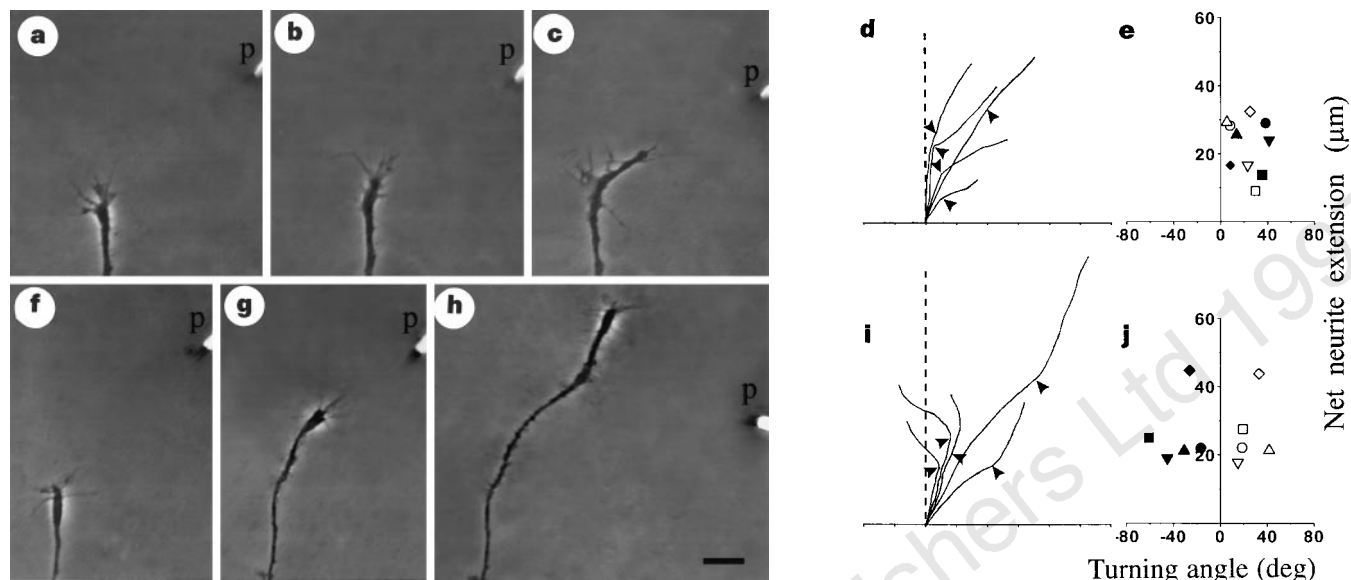


Figure 3 Switching from attractive to repulsive turning induced by KT5720. **a–c**, A neuron was exposed to the same BDNF gradient as for Fig. 1 for 1 h, followed by exposure to the same gradient for another hour with the pipette repositioned to a site 100 μm away and oriented 45° with respect to the new neurite direction, and KT5720 (200 nM) was added to the saline. **a**, At the onset; **b**, end of the first hour; **c**, end of the experiment. Tick marks, 10 μm . **d**, Superimposed traces of the

trajectory of neurite extension in five neurons examined as that described in **a–c**. Triangles mark the end of the first hour. **e**, Scatter plot of the net neurite extension and final angular position of the growth cone at the end of the first (open symbols) and second hour (filled symbols). **f–j**, The same as that described in **a–e**, except that no drug was added into the saline during the second hour.

responses induced by BDNF require Ca^{2+} influx into the growth cone.

We also examined whether modulation of cAMP-dependent activity results in a switch between attractive and repulsive responses to the BDNF gradient in the same neuron. First we applied the standard BDNF gradient for 1 hour in normal saline, which induced attractive turning of the growth cone; the BDNF pipette was then repositioned 100 μm away and at a 45° angle with respect to the new direction of neurite extension for a further hour, either in the presence or absence of KT5720 (200 nM). As shown in Fig. 3a–e, there was a consistent switch to the repulsive response in the presence of KT5720, whereas there was further attractive turning in the absence of the drug (Fig. 3f–j). These results also indicate that the BDNF gradient did not desensitize the growth-cone response. The switch of the turning behaviour with KT5720 occurred without alteration in the rate of neurite growth (Fig. 3d,e) and both attractive and repulsive responses were preceded by asymmetric filopodia protrusion activity on two sides of the growth cone. No collapsing effect was observed after addition of KT5720.

The cultured *Xenopus* neurons also show attractive turning towards the source of neurotrophin-3 (NT-3), which acts through a different receptor (TrkC) from that used by BDNF (TrkB). Reduction of $[\text{Ca}^{2+}]_0$ to 1 μM , or addition of Rp-cAMPS (20 μM) was ineffective in altering the attractive response induced by NT-3 (Fig. 2e,f; Table 1). Thus the cAMP switch of the turning behaviour may operate only in neurons for specific types of guidance signals. The dependence of the effects of BDNF (but not of NT-3) on cAMP was also found for the long-term survival and growth of developing retinal ganglion cells in culture²¹. Gradients of ACh are known to be attractants for these *Xenopus* growth cones¹³: the transmitters appear to act by opening receptor channels to allow an influx of Ca^{2+} into the growth cone; the turning response is prevented by removing extracellular calcium¹³. Here we find Rp-cAMPS (20 μM) in the bath causes a repulsive turning of the growth cone in the presence of an ACh gradient (Fig. 2g,h; Table 1). The similar requirement for extracellular Ca^{2+} in ACh- and BDNF-induced turning responses, together with the findings that both ACh and

BDNF can trigger an increase in cytosolic Ca^{2+} in these neurons^{13,22}, suggests that the cAMP switch may operate in the turning induced by Ca^{2+} influx. The concentration of calcium in the neuron can influence the production of cAMP in the neuron through activation of Ca^{2+} -dependent adenylyl cyclase^{23,24}. An extracellular gradient of BDNF or ACh may trigger a cytoplasmic Ca^{2+} gradient, which leads to a cAMP gradient within the growth cone and causes an attractive turning response¹². On the other hand, elevation of cytosolic Ca^{2+} also inhibits growth-cone motility and neurite extension¹⁵, so the cytosolic Ca^{2+} gradient by itself may induce a repulsive response in the growth cone; this effect is normally overridden by the attractive response owing to the cAMP gradient generated by the Ca^{2+} gradient. Inhibition of cAMP-dependent processes by Rp-cAMPS or KT5720, which block the attractive response, may thus reveal the repulsive effect of the cytosolic Ca^{2+} gradient. cAMP signalling presumably follows an increase in Ca^{2+} concentration, as a forskolin gradient can still induce an attractive response in a low- Ca^{2+} medium (Table 1).

A family of proteins known as netrins act as attractants for axons extending towards the ventral midline of the nervous system and as repellents for axons growing away from it^{10,25–27}. Different netrin receptors may induce these different responses by modulating cAMP levels in the neuron. The action of transmitters and modulators is often mediated by regulation of second messengers^{20,28–30} like cAMP. The presence or absence of these factors may thus switch the growth cone's response to the same guidance cue between attractive and repulsive. During its pathfinding in the developing embryo, a growth cone may be attracted to a 'guidepost' cell by a gradient of diffusible factor released by the cell. Upon arrival at the guidepost cell, changes in the cAMP level due to the presence of another signal, perhaps triggered by the contact of the guidepost cell, may switch the attractive response to a repulsive one, allowing the axon to grow away from the cell for its next stage of pathfinding. Finally, electrical activity can alter the level of cAMP in the active neuron through the activation of Ca^{2+} -dependent adenylyl cyclase^{23,24} and also in nearby neurons through the action of neuromodulators released by the active neuron³⁰. Activity-dependent

competition and refinement of nerve terminals near the target neuron may thus involve attraction or repulsion of the target-derived factor on the active and inactive nerve terminals, respectively, depending on their cAMP levels. □

Methods

Cell cultures. Cultures of *Xenopus* spinal neurons were prepared from the neural tube tissue of 1-day-old *Xenopus* embryos as described^{12,13}. The tissue was dissociated in Ca^{2+} - and Mg^{2+} -free solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 0.5 mM EDTA, pH 7.4) for 20 min and plated on the surface of clean glass coverslips. The culture was preincubated at room temperature (20–22°C) for 14 h before use. The culture medium consisted of 49% (v/v) Leibovitz medium (GIBCO), 1% (v/v) fetal bovine serum (HyClone) and 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM CaCl_2 , 2.5 mM KCl, 10 mM HEPES, pH 7.4). The experiments were carried out in modified Ringer's solution (140 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 5 mM KCl, 10 mM HEPES, pH 7.4). Low- Ca^{2+} (1 μM) medium was modified Ringer's solution containing 0.9 mM CaCl_2 , 4 mM EDTA.

Production of microscopic chemical gradients. Microscopic gradients of chemicals were produced as described^{12,13}. Briefly, repetitive pressure injection of picolitre volumes of solutions containing the chemical was applied through a micropipette that had a tip opening of $\sim 1 \mu\text{m}$. The pressure was applied with an electrically gated pressure application system (Picospritzer General Valve). A standard pressure pulse of 3 p.s.i. in amplitude was applied for 20 ms to the pipette at a frequency of 2 Hz using a pulse generator (SD9, Grass Instruments). By measuring the size of droplet in the mineral oil after 50 pulses of repetitive ejection with the same pressure pulse parameters, the average volume of ejected solution per pulse was estimated to be $\sim 1.5 \text{ pl}$. Analysis of the gradient^{12,13} indicates that, under these pulse parameters, the average concentration of the chemical was $\sim 10^3$ -fold lower than that in the pipette, and a concentration gradient of ~ 5 –10% was created across a growth cone $10 \mu\text{m}$ wide, $100 \mu\text{m}$ away from the tip of the ejecting pipette. The total volume of the saline in the culture dish during the experiment was 5 ml. The final average bath concentration of BDNF at the end of the 1-h experiment was $\sim 0.1 \text{ ng ml}^{-1}$.

Measurements of extension and turning of the growth cone. A phase-contrast inverted microscope (Nikon Diaphot) was used to observe the neurite growth. Microscopic images of the neurite were recorded with a CCD camera (Toshiba IK-541RA) attached to microscope and stored on videotape. To find the length of neurite extension, the entire trajectory of the neurite at the end of an hour was measured with a digitizer. To assay growth cone turning, the tip of the pipette containing the chemical was placed $100 \mu\text{m}$ away from the centre of the growth cone and at an angle of 45° with respect to the direction of initial direction of neurite extension. The direction of the neurite was determined by the last $10\text{-}\mu\text{m}$ segment of the neurite. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the 1-h period. Only growth cones with a net extension $> 5 \mu\text{m}$ over the 1-h period were scored for the turning assay.

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Synapse specificity of long-term potentiation breaks down at short distances

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Long-term potentiation (LTP), the long-lasting increase in synaptic transmission, has been proposed to be a cellular mechanism essential for learning and memory, neuronal development, and circuit reorganization. In the original theoretical¹ and experimental² work it was assumed that only synapses that had experienced concurrent pre- and postsynaptic activity are subject to synaptic modification. It has since been shown, however, that LTP is also expressed in synapses on neighbouring neurons that have not undergone the induction procedure^{3–5}. Yet, it is still believed that this spread of LTP is limited to adjacent postsynaptic cells, and does not occur for synapses on neighbouring input fibres^{2,6,7}. However, for technical reasons, tests for 'input specificity' were always done for synapses relatively far apart. Here we have used a new local superfusion technique, which allowed us to assess the synaptic specificity of LTP with a spatial resolution of $\sim 30 \mu\text{m}$. Our results indicate that there is no input specificity at a distance of less than $70 \mu\text{m}$. Synapses in close proximity to a site of potentiation are also potentiated regardless of their own history of activation, whereas synapses far away show no potentiation.

Synaptic specificity of LTP was examined for synapses between the Schaffer collaterals and the CA1 region in organotypic slice cultures of rat hippocampus^{3,8}. By using a local superfusion technique⁹ we localized and individually activated neighbouring groups of synapses with a spatial resolution of less than $30 \mu\text{m}$.

A pyramidal neuron in the CA1 region of the hippocampus was recorded intracellularly, and an excitatory postsynaptic potential (EPSP) was evoked by stimulation of the Schaffer collaterals (Fig. 1a). Subsequently, transmitter release and hence all EPSPs (and inhibitory postsynaptic potentials) were blocked in the whole culture by replacing the normal extracellular medium with a solution containing a low calcium concentration (0.8 mM) and 10 μ M cadmium. Synaptic transmission was then restored locally by superfusing a small area of $\sim 30 \mu$ m diameter (Fig. 1c) with normal medium containing a slightly elevated calcium concentration (5 mM; see Methods for explanation), no cadmium and a visible dye. The superfusion pipettes were moved along the dendritic arbor of the postsynaptic cell until a group of synapses was found that mediated a small EPSP when the Schaffer-collaterals were stimulated (Fig. 1b).

After one group of active synapses (the 'control' group) was located (Fig. 1d), its vicinity (40–150 μ m) was searched with the superfusion spot, perpendicular to the Schaffer collaterals, to find

another group of synapses (the 'test' group) through which the postsynaptic cell could also be activated (Fig. 1e). LTP was then induced in the test group by pairing postsynaptic depolarization with the presynaptic stimulus (Fig. 1f). Finally, to test whether the control group of synapses was affected by induction of LTP in the neighbouring test group, the superfusion spot was moved back to the control site (Fig. 1g) and these synapses were tested again. Any increment in the control EPSP amplitude would indicate a non-specific spread of LTP from the test group, as there was no synaptic input activating the control group while the pairing was applied.

To ensure that there was no cross-talk between the two superfusion positions, we performed a control using only the *N*-methyl-D-aspartate (NMDA) component of the EPSPs. This was done for two reasons: (1) it allowed us to make use of the irreversible open-channel blocker MK-801 (ref. 10), and (2) we could thereby test the component of the EPSP that is presumably responsible for the induction of LTP. NMDA responses were isolated by exchanging the superfusion solution to one containing DNQX (10 μ M) and no

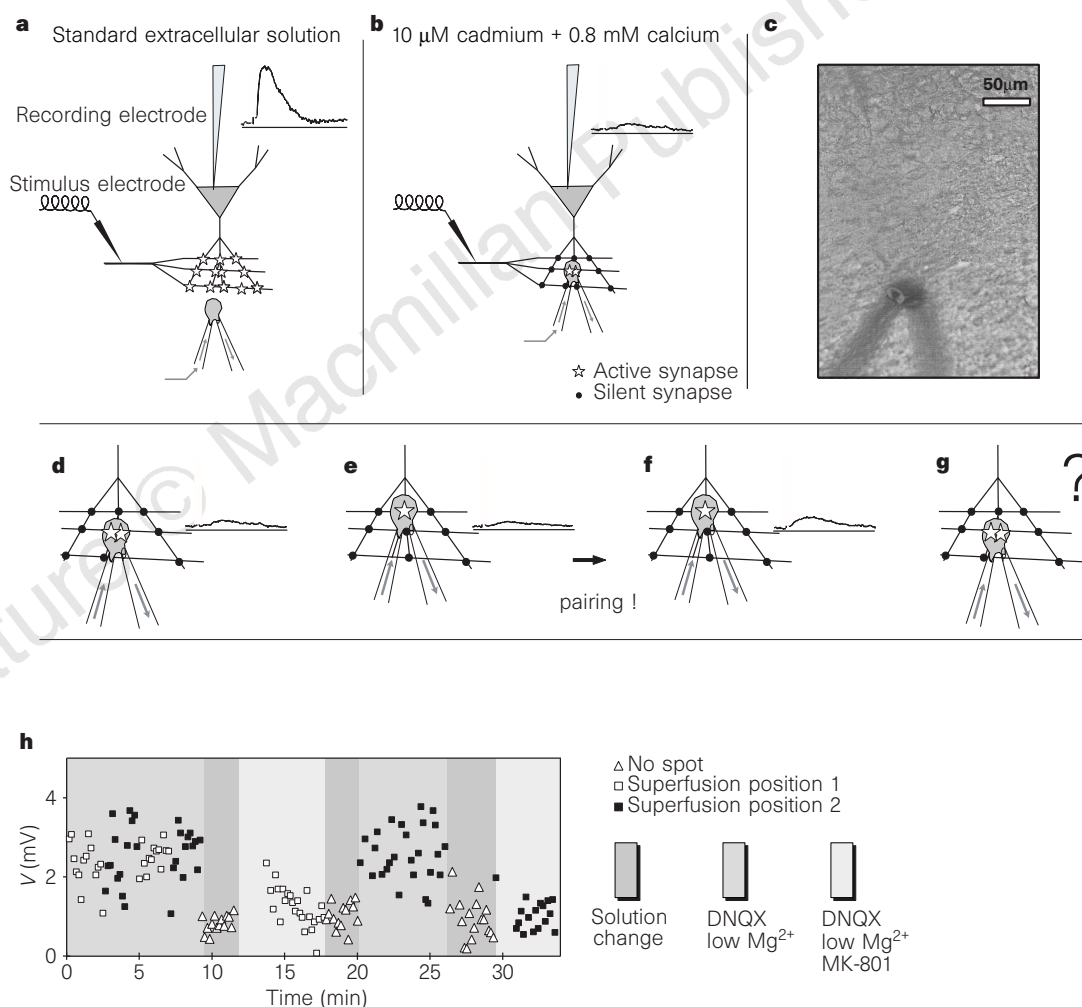


Figure 1 Schematic representation of experimental approach. **a, b**, Schematic representation of the recording situation. Actual EPSPs are shown as insets in the top right-hand corners. Active synapses are depicted as stars, synapses blocked by 10 μ M Cd^{2+} and low Ca^{2+} as black dots. **c**, Micrograph of the superfusion spot on the slice culture showing that the size of the spot can be limited to about 30 μ m. The recording electrode can be seen in the top left-hand corner; the application pipette and the shadow of the suction pipette as well as the dyed superfusion solution are seen in the bottom half. **d-g**, Sequence of events in an actual experiment (**d** is an enlargement of part of **b**). Further explanations see text. **h**, the MK-801 test demonstrates that there is no overlap between the two groups of

activated synapses. Different shadings symbolize different solutions in the superfusion pipette. Filled and open squares show responses recorded while superfusion was applied to synapses of group 1 or group 2. After application of MK-801 to group 1 the responses are at noise level (at ~ 16 min; noise level can be judged from the open triangles, which show data collected when the superfusion spot was removed from the culture for purposes of solution change). When the superfusion solution is changed back to medium without MK-801, and is applied to group 2 (20–26 min), responses have not changed compared to the control period (0–9.5 min), indicating that there was no activity in group 2 when group 1 was superfused.

Mg²⁺ (Fig. 1h; 0–9.5 min). Synaptic transmission in the non-superfused part of the culture was again blocked by Cd²⁺ and low Ca²⁺ concentration. We then eliminated the test responses entirely by superfusing these synapses with medium containing MK-801 (Fig. 1h; 12–18 min) and went back to the control position, again with no MK-801 in the superfusion, to check whether the responses of these synapses had been affected (Fig. 1h; 20–26 min). There was no change in amplitude, indicating that there was no cross-talk between the test and control synapses; a change in amplitude would have indicated that there was an overlap between the two groups. Even the argument that Ca²⁺ diffuses differently from MK-801 can be discounted because any extended diffusion of Ca²⁺ would have prevented us from being able to block the activity fully while superfusing the test position. In none of the control experiments ($n = 10$, distances between 40 μm and 70 μm) did we observe a cross-talk between control and test site. Thus it was possible to isolate reliably and activate individually groups of synapses that were 40 μm or more apart, allowing us to take a fresh look at input specificity.

Our experiments addressing this issue followed the scheme shown in Fig. 1d–g; the results of our first experiment are shown in Fig. 2a. After establishing a baseline for the two superfusion positions which were separated by 50 μm (test and control-50; white and black squares, respectively), the pairing procedure was applied and LTP was induced while synaptic activity was restricted to the test position (note the increasing synaptic responses between 0 and 8 min; white squares). After LTP had been firmly established, the superfusion spot was moved to the control position, and to our surprise, enhancement was also observed for these synapses (Fig. 2a, black squares; 9–12 min and thereafter), despite their lack of activity during the pairing procedure. This was the first demonstration that LTP can spread along the postsynaptic dendrite from activated synapses to nearby synapses that had not undergone the pairing procedure.

To estimate the inter-synaptic distances over which this spreading occurs, we performed recordings where, within a single experiment, the spot was positioned at distances of 50 μm and 100 μm from the

test site. This experiment (Fig. 2b) showed that, although the control synapses at a distance of 50 μm (control-50, black squares) were again clearly potentiated, there was no such increase at a distance of 100 μm (control-100, grey squares). This demonstrates that the effect has a range of between 50 and 100 μm , and at the same time proves that we were not observing nonspecific changes, such as changes in general excitability. In this particular experiment (and in five of the eight experiments in which the intersynaptic distance was such that LTP was elicited in both control and test synapses) we also performed the MK-801 control (Fig. 2b; 45–83 min) immediately after the test for input specificity. This control showed that the group of synapses at 50 μm could not have experienced any kind of synaptic activity during the induction of LTP in the test group. All of the other experiments of this series are shown in Fig. 3a. It is evident that there is no effect on the control group when the distance between the groups of synapses is greater than 70 μm , whereas for every single experiment in which it was smaller or equal to this number, the effect was always significant (in all cases $P < 0.0001$; Students t -test). The ratio of the enhancements for control and test group are plotted against distance for all experiments in Fig. 3b, substantiating our conclusion that the range of the spread of LTP is limited to ~ 70 μm from the site of induction.

To confirm our data using a different experimental approach we performed another series of experiments in which two stimulating electrodes (again termed test and control) were positioned as far apart as possible to ensure that two independent axon bundles were stimulated (Fig. 4a). A slightly larger superfusion spot of ~ 60 μm diameter was used as we were now searching for an area on the dendritic tree with synapses between both stimulated axon bundles and the postsynaptically recorded neuron. After having found such an area we performed 'collision tests'¹¹ to verify that the synapses within this confined area were indeed from different and separable inputs. After recording a baseline (with alternating stimulation), postsynaptic depolarization of the recorded neuron was paired only with stimuli of the test electrode, and we examined whether only the test synapses, or also the nearby control synapses, showed enhancement. Also with this experimental approach we found a synaptic

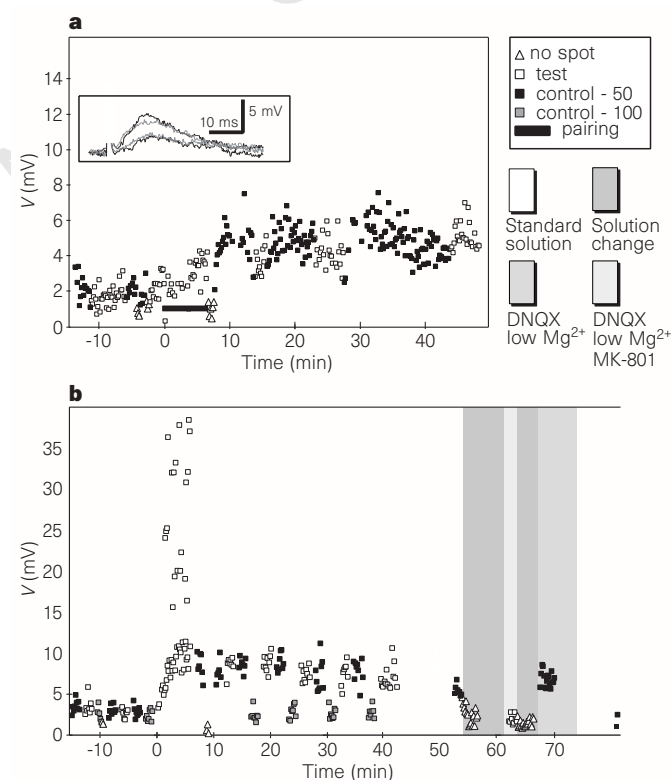


Figure 2 Demonstration that LTP can spread from activated synapses to nearby synapses that have not undergone the pairing procedure. **a**, After establishing a baseline for the two superfusion positions (test, white squares; control-50, black squares), pairing was applied (black bar) while the synaptic activity was restricted to the test position. After LTP had been established in the test synapses, the superfusion spot was moved to the control-50 position (50 μm from the test position) and a notable and long-lasting enhancement of the EPSP was also observed for these synapses (black squares, 9–12 min and thereafter). The inset shows typical responses directly before and ~ 30 min after the pairing. The thin traces correspond to the white squares (test position), the thick traces to the black squares (control-50 position). **b**, The same kind of experiment as in **a**, but a third group (control-100, grey squares) at a distance of 100 μm was also examined. Although control-50 was again increased, there is no significant change of EPSP amplitude before or after the pairing procedure (before, 2.4 ± 0.7 mV; after, 2.6 ± 0.7 mV (mean $\pm$ s.d.) in control-100. The very large responses between 0 and 10 min are caused by action potentials that occurred occasionally during the pairing procedure. In this experiment an MK-801 control (45–83 min) was performed immediately following the actual experiment.

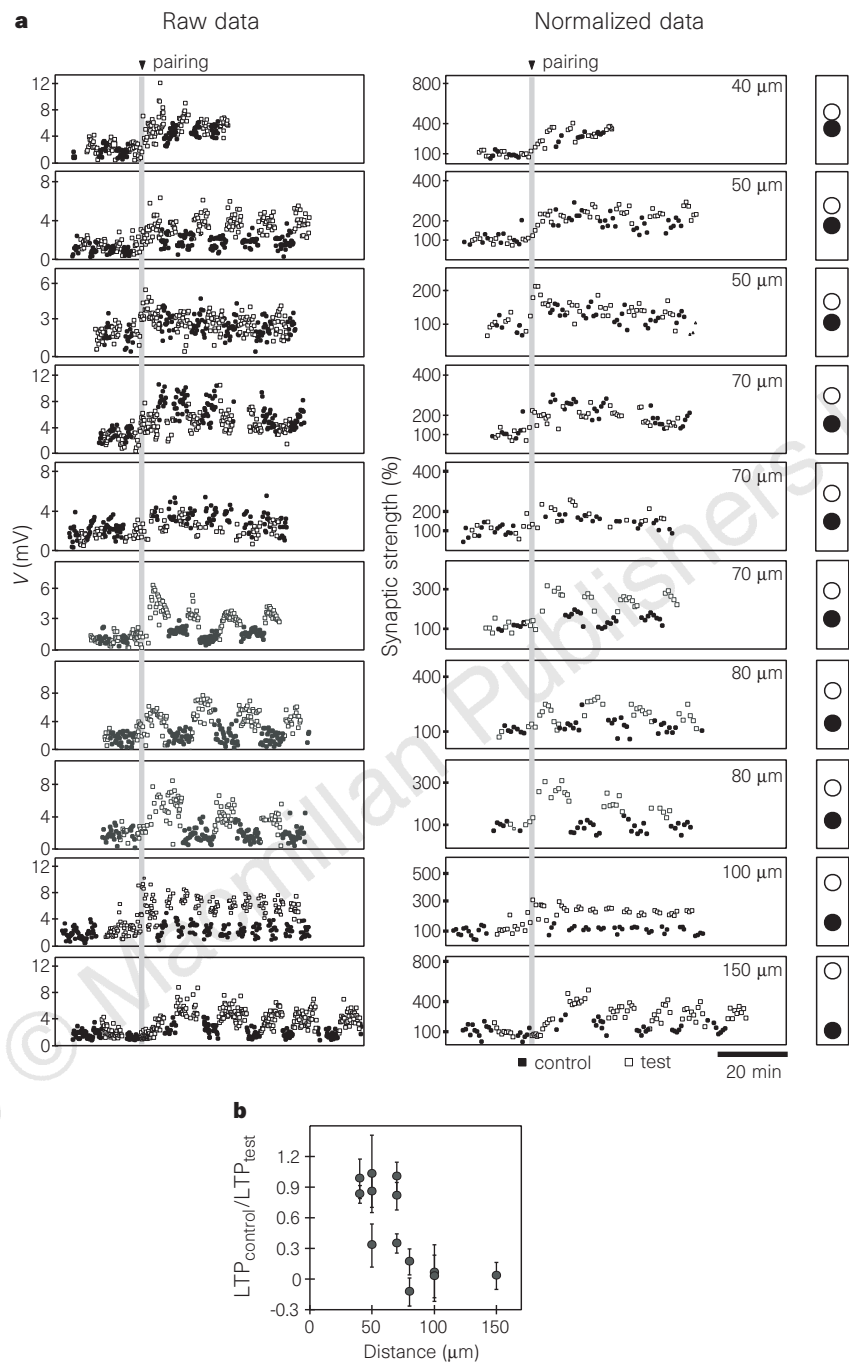


Figure 3 Complete data set for these experiments (excluding those already displayed in Fig. 2). **a**, Raw data are shown on the left, and data normalized to baseline for test and control group separately, averaged over 5 data points, are shown on the right. The two circles on the extreme right indicate the distance between the test and control group for each experiment. No effect on the control group can be observed when this distance was greater than 70 μm (bottom four panels of left and right columns), whereas a significant increase can be seen in all other cases. **b**, Ratio of the enhancements for control and test group plotted

against the distance for all experiments. To obtain a meaningful ratio for the two values we defined, only for the purpose of this figure, no change in synaptic strength (usually 100%) was 0% LTP; a doubling was 100%. Signal size for the calculation of LTP was measured usually 30 min after the beginning of the pairing procedure and averaged over 10 consecutive sweeps. One exception was the cell of the two top panels in **a**, for which signal amplitude was measured at 25 min. Error bars indicate standard error.

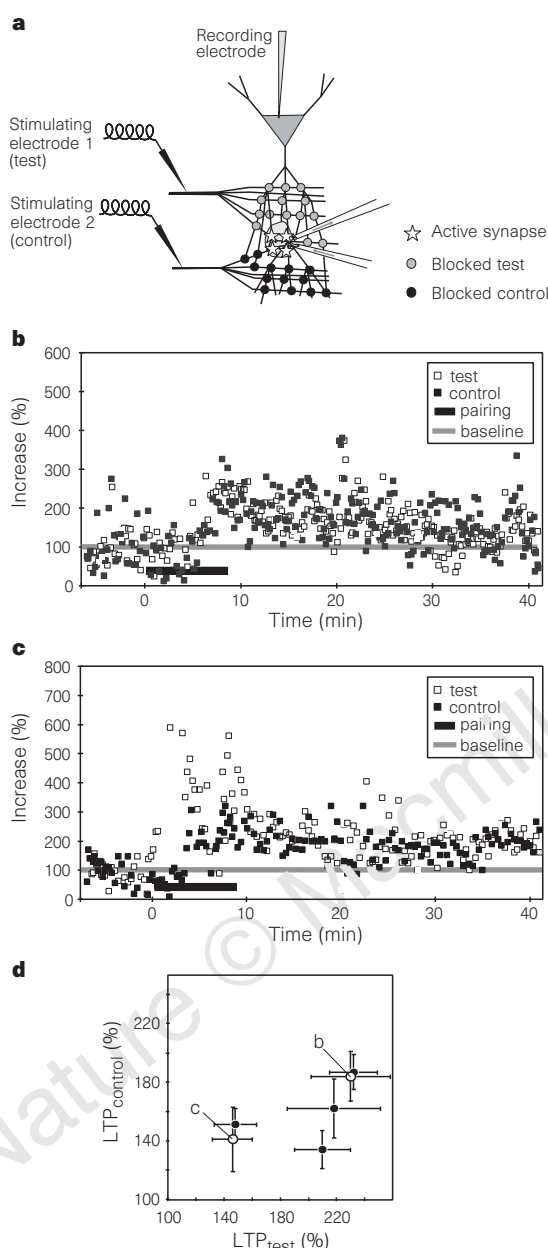


Figure 4 Lack of input specificity shown using a different approach. **a**, Schematic representation of the experimental approach: two stimulating electrodes were positioned at the two borders of the stratum radiatum and pharmacological isolation was used to isolate an area of $\sim 60\mu\text{m}$ in diameter that contained synapses between both stimulated fibre bundles and the postsynaptically recorded neuron (active synapses are depicted as white stars, blocked synapses as grey and black dots, for the test and control pathway, respectively). In both experiments shown (**b** and **c**), increases in the control pathway are of similar size to that in the test pathway. **d**, Quantitative results for all six such experiments obtained by plotting increase for the control pathways against increase for the test pathways (the experiments of **b** and **c** are depicted as open circles). Mean and standard error were calculated for 10 sweeps at 30 min.

enhancement in the control pathway of similar size to the one in the test pathway (Fig. 4b,c). Sometimes there was a clear delay of onset in the potentiation of the control pathway (~ 5 min in Fig. 4c; other delays ranged between 0 and 5 min), further supporting that the two pathways were independent. The quantitative results for all six such experiments (Fig. 4d) show that, in every experiment, the potentiation in the test pathway was always accompanied by a significant potentiation of the control pathway.

Previous experiments have reported that LTP is input specific. However, owing to the relatively high spatial divergence of the Schaffer collaterals, these experiments had to be performed by stimulating two fairly distant pathways^{2,6,12}, and so they did not allow assessment of the specificity of LTP in the range of tens of micrometres. Our method of 'pharmacological isolation' allowed us to tackle this problem in two ways. In the first series of experiments, we could differentiate between synapses that were very close together while being able, by using the MK-801 control, to show that one site was inactive while LTP was induced in the other. Our second series of experiments allowed us, very much in line with earlier studies¹³, to address input specificity by stimulating two pathways. Our specific advantage was that the superfusion technique allowed us to isolate those synapses that are most affected by the spreading of LTP, disregarding the large number of synapses that would, with their irrelevant contribution, otherwise obscure the relatively small effect.

A retrograde messenger has been postulated^{14–16} to mediate between the postsynaptic induction of LTP and its at least partly presynaptic expression^{17–20}. Such a messenger would account for the presynaptic spreading of enhancement^{3–5} but, because it was assumed that LTP was input specific, it had to be postulated that the retrograde messenger would only be effective on activated presynaptic terminals¹⁴. This view has been questioned by reports that presynaptic activity is not necessary, and that artificial postsynaptic elevation of calcium alone is sufficient to induce an LTP-like enhancement^{21,22}. Our results confirm and extend this finding by showing that, also for conventional LTP (as induced by the pairing procedure), presynaptic activity is not a requirement for the expression of LTP. Whereas our first series of experiments demonstrates that presynaptic transmitter release is not required for LTP, the second series shows that not even electrical activity in the presynaptic fibres is necessary to produce synaptic enhancement.

The concept of extracellular^{7,15,16} and intracellular²³ messengers communicating between neighbouring synapses has recently received increasing attention. We are unable to say conclusively whether the spread of synaptic enhancement reported here is mediated by an extracellular or an intracellular mechanism. However, if an intracellular substance were to mediate the spreading enhancement we would have expected to see occasional failures of this phenomenon in our first series of experiments. These should have occurred if two groups of synapses less than $70\mu\text{m}$ apart would have come to lie on different dendritic branches, resulting in a 'cytoplasmic distance' considerably greater than $70\mu\text{m}$. If an intracellular substance were to mediate the spread one might be concerned that a different synaptic morphology in slice cultures could result in less postsynaptic 'compartmentalization', possibly leading to the effect observed here. This seems unlikely, as previous studies in hippocampal²⁴ and neocortical²⁵ slice cultures have reported the structure of spines, as well as the ratio of shaft to spine synapses, to be largely normal. Moreover, we have examined our cultures electronmicroscopically in that respect (data not shown), and have not observed any abnormalities.

In summary, our two series of experiments demonstrate that input specificity of LTP is not sustained below $70\mu\text{m}$. However, our data are also compatible with previous reports^{2,6,12} in that they show that enhancement is not totally nonspecific: for distances greater than $70\mu\text{m}$, synaptic enhancement remains restricted to the

synapses activated during the induction procedure. Taken together with earlier studies^{3–5}, our data suggest that the strict concept of a Hebbian synapse has to be modified to encompass the notion of enhancement spreading several tens of micrometres from the initiation site. If one believes that the Hebbian mechanism is the basis for information storage in the brain then our results indicate that for information storage—not necessarily for information processing—groups of synapses, rather than single synapses, are the basic computational unit. □

Methods

Slice cultures. Organotypic slice cultures of rat hippocampus were prepared⁸ and selected for recording after 2–4 weeks in culture. Monolayered slice cultures were used because the penetration depth of the superfusion technique, which is central to these experiments, is not much more than ~30–50 μm , so using this technique in acute brain slices is impractical.

Data acquisition and analysis. Intracellular recordings were performed with an AxoClamp-2B amplifier (Axon Instruments) in current-clamp configuration. Sharp electrodes were used to prevent rundown of cells caused by washout of intracellular factors. Data were low-pass filtered at 3 kHz, digitally sampled at 5 kHz, and stored and analysed with custom-made LabView Software (National Instruments). In the figures the amplitude of the EPSP (in mV) is plotted over time. The maximal slopes of EPSPs were also determined in all experiments, but the results are virtually the same (apart from the expected slightly increased scatter of the data points), and so they are not shown.

Electrophysiology. Monopolar stimulus electrodes were made from tungsten wire (TW-8-3, Science Products, Hofheim, Germany), electrolytically sharpened and coated with resin (EPR-4, Clark, Pangbourne, Reading) under microscope control. Recording electrodes were pulled from borosilicate glass and filled with 2 M potassium-methyl-sulphate, resulting in a final resistance of 80–120 M Ω . Modified Hanks balanced salt solution (HBBS) containing 3.2 mM Ca^{2+} and 0.9 mM Mg^{2+} was used as the standard extracellular recording solution. To block synaptic transmission, Ca^{2+} was reduced to 0.8 mM and cadmium (CdCl_2) in a very mild concentration of 10 μM was added (to block voltage-activated calcium channels). Synaptic function was restored locally using a superfusion technique.

LTP and pairing procedure. Only cells with a resting membrane potential below –65 mV were considered. LTP was verified to be standard in the organotypic cultures by showing that tetanus-induced changes were invariably blocked by the selective agonist AP5 (50 μM ; data not shown), and that neither postsynaptic depolarization nor presynaptic low-frequency stimuli alone induced LTP (data not shown). The pairing procedure used to induce LTP consisted of 200 ms postsynaptic current injections of 1.5 nA paired with single presynaptic stimuli applied 5 ms after start of depolarization. To monitor the change of synaptic strength during the pairing procedure, 30 repetitions at 0.1 Hz were interleaved with unpaired presynaptic stimuli. For the second series of experiments, stimuli were applied every 5 s, first the paired test stimulus then, consecutively, test and control unpaired (again, 30 repetitions).

Superfusion. The superfusion device is described in detail elsewhere⁹. It consisted of two glass pipettes with tip openings of ~15 μm placed opposite each other with an inter-tip distance of ~20 μm . One pipette served for solution delivery and could be connected to different reservoirs located at a slightly elevated position to provide gravitational pressure to the outflowing solution. Change from one reservoir solution to another was made faster by an additional suction needle in the dead space of the pipette that served to evacuate the interior of the pipettes quickly and enabled the speedy replacement by the solution from the next reservoir. It was therefore possible to use more than one superfusion solution without changing the position of the pipette or the size of the superfused area. The second glass pipette was connected to a peristaltic pump that removed the superfusion solution from the external medium, thereby reducing the affected area to a spot of as little as 30 μm in diameter. Size and position of the spot were made visible using food colour in the superfusion solution (see Fig. 1c); the colour has no adverse effects on the electrophysiological properties of the neurons⁹. The two pipettes were mounted on a custom-made set of manipulators to adjust them towards each other before the experiment. These manipulators were themselves mounted on a computer-programmable manipulator (Luigs and Neumann,

Ratingen, Germany), which was used to move the whole arrangement in three dimensions during the recordings. The precision in (re-)positioning the spot was estimated to be <1 μm .

In the superfusion solution a Ca^{2+} -concentration of 5 mM was used. It is important to consider that the concentration in the superfusion solution is significantly diluted until it has reached the synaptic terminals deep in the tissue. The actual concentration within the tissue was probably somewhere between 1 and 4 mM. Because a minimal concentration of 5 mM in the superfusion solution was necessary to enable synaptic transmission locally (in more than 30% of cells), the true concentration at the synaptic sites was probably closer to 1 mM than to 4 mM. The superfusion onto the synapses in the tissue was rather fast. We found that synaptic potentials could be observed ~30 s after moving the superfusion spot to the correct position. These potentials vanished ~20 s after the spot was removed (see Fig. 1h; open triangles show records in which the spot was removed).

MK-801 control. The MK-801 control for overlap between the different groups of synapses was included in five of the eight experiments in which LTP had spread to the control site. It was also done on five cells that did not exhibit LTP in the test group but could conveniently be used to test the confinement of the superfusion spot. To isolate the NMDA component of the EPSP, 10 μM DNQX was added to the superfusion solution and Mg^{2+} was removed. To block the NMDA component irreversibly, 100 μM MK-801 (Research Biochemicals Incorporated) was added to the solution.

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Molecular assessment of the potential transmissibilities of BSE and scrapie to humans

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More than a million cattle infected with bovine spongiform encephalopathy (BSE) may have entered the human food chain¹. Fears that BSE might transmit to man were raised when atypical cases of Creutzfeldt–Jakob disease (CJD), a human transmissible spongiform encephalopathy (TSE), emerged in the UK^{2,3}. In BSE and other TSE diseases, the conversion of the protease-sensitive host prion protein (PrP-sen) to a protease-resistant isoform (PrP-res) is an important event in pathogenesis^{4–7}. Biological aspects of TSE diseases are reflected in the specificities of *in vitro* PrP conversion reactions^{8–12}. Here we show that there is a correlation between *in vitro* conversion efficiencies and known transmissibilities of BSE, sheep scrapie and CJD. On this basis, we used an *in vitro* system to gauge the potential transmissibility of scrapie and BSE to humans. We found limited conversion of human PrP-sen to PrP-res driven by PrP-res associated with both scrapie (PrP^{Sc}) and BSE (PrP^{BSE}). The efficiencies of these heterologous conversion reactions were similar but much lower than those of relevant homologous conversions. Thus the inherent ability of these infectious agents of BSE and scrapie to affect humans following equivalent exposure may be finite but similarly low.

Experimental transmission of BSE to mice¹³, cattle¹⁴ and two sheep PrP genotypes (A136 Q171 (ov-AQ) and V136 Q171 (ov-VQ))¹⁵ has been reported, but hamsters (J. D. Foster and J.H., unpublished data) and at least one PrP genotype of sheep (A136 R171 (ov-AR))¹⁶ seem to be resistant to clinical disease. To test for a correlation between *in vitro* cell-free conversion and *in vivo* transmission, we included types of ³⁵S-labelled PrP-sen from each susceptible or resistant group of species in PrP^{BSE}-driven conversion experiments. ProteinaseK-resistant ³⁵S-labelled conversion products that were the characteristic 6K to 7K smaller than the ³⁵S-PrP-sen substrate^{8–12,17} were generated in the reactions with both glycosylated and aglycosyl ³⁵S-PrP-sen molecules from BSE-susceptible, but not BSE-resistant, hosts (Fig. 1). For example, the 25K aglycosyl bovine and sheep (ov-AQ and ov-VQ) ³⁵S-PrP-sen proteins generated 18K ³⁵S-PrP-res bands (Fig. 1, lanes 1, 6, 7, 13, 15, 16), whereas the aglycosyl hamster and ov-AR gave no proteinaseK-resistant product (Fig. 1, lanes 17, 20, 21). Thus, the efficiency of the PrP^{BSE}-induced conversion of PrP-sen of a given host was found to

be correlated with the *in vivo* transmissibility of BSE to that host. This correlation encouraged us to look for an *in vitro* indication of the transmissibility of BSE to humans, using a PrP^{BSE}/human PrP-sen conversion assay.

Wild-type human (h) PrP has two common allelic forms that encode either methionine (hPrP-M) or valine (hPrP-V) at codon 129 (ref. 18). Hence we tested both types of hPrP-sen in conversion experiments. PrP^{BSE} converted the ~25K aglycosyl forms of ³⁵S-hPrP-M and ³⁵S-hPrP-V to ~18K proteinaseK-resistant forms compatible with the proteinaseK-resistant core of PrP found in the human diseases (Fig. 1, lanes 2, 3, 4, 14; Fig. 2, lane 8). When more glycosylated ³⁵S-hPrP-M was used, the proteinaseK-resistant conversion product was still largely restricted to an ~18K band, suggesting preferential conversion of the 25K aglycosylated form of ³⁵S-hPrP-M (Fig. 1, lane 9). Little or no spontaneous formation of proteinaseK-resistant PrP was observed in the absence of PrP-res (Fig. 1, bottom). The ³⁵S-hPrP-V labels were converted by PrP^{BSE} roughly threefold less efficiently than the ³⁵S-hPrP-M labels (Fig. 1, lanes 2–5, 9, 10; Fig. 2, lanes 8, 9; Fig. 4; *P* = 0.0025). So far the new variant CJD has been found only in patients homozygous for methionine at codon 129 (ref. 2) and, although it may be premature

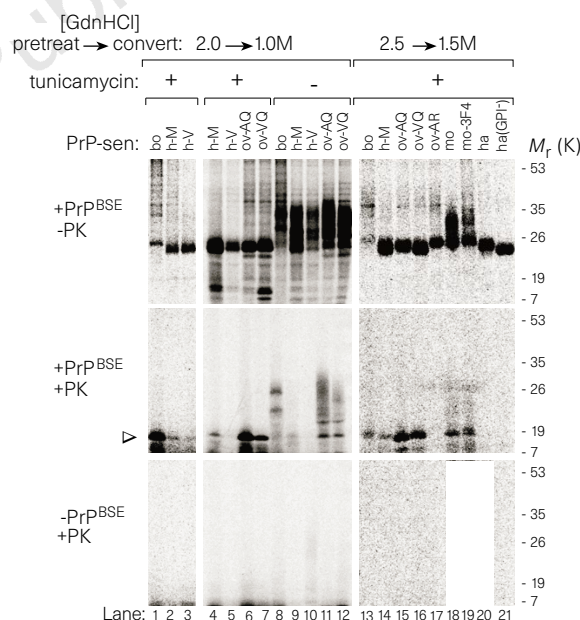


Figure 1 Phosphor autoradiographic analysis of SDS-PAGE gels of cell-free conversion products formed on incubation of PrP^{BSE} with ³⁵S-PrP-sen from different species. The molecular size range of ³⁵S-radiolabelled PrP proteins following incubation with PrP^{BSE} is seen before (–PK) or after (+PK) digestion with proteinase K in the top two panels (+PrP^{BSE}, –PK and +PrP^{BSE}, +PK). The effect of omitting PrP^{BSE} from the incubation is shown in the bottom panels (–PrP^{BSE}, +PK). The migration of *M_r* markers is shown on the right. The ~18K PK-resistant ³⁵S-PrP product is marked by the arrowhead. Equal amounts of PrP^{BSE} (~0.5 µg) were used in each reaction with an approximately equivalent amount of radioactive PrP-sen (~25,000–40,000 c.p.m. per reaction; apart from boPrP-sen reactions, where ~10,000 c.p.m. was used owing to inefficient labelling in the bovine cells). The reaction sample was divided for analysis ~1:10 (–PK: +PK) between the gels shown in the top two panels (and also between the top and bottom panels). Results are shown using differing concentrations of GdnHCl in the pretreatment and conversion phases of the reaction (see Methods). Conversions using ³⁵S-PrP-sen molecules labelled in the presence (+) or absence (–) of tunicamycin are shown. The relative intensities of bands in the +PrP^{BSE}, +PK panel need not reflect the efficiency of conversion as the specific activities of each label are not always the same; the percentage of label converted to proteinase K-resistant fragments (summarized in Fig. 4) is a better indicator of this efficiency than absolute intensities of the PK-resistant bands in the +PK, +PrP^{BSE} panel.

to speculate on the basis of these conversion data, such genotypic selection may result from a more efficient conversion of hPrP-M than hPrP-V by PrP^{BSE}.

To help gauge the efficiency of these various PrP^{BSE}-induced reactions, we compared these data with the proportion of labelled hPrP-sen (M or V) converted by a similar amount of human PrP^{CJD} isolated from either new variant (Figs 2, 4) and sporadic CJD (not shown) patients homozygous for methionine at codon 129 (PrP^{CJD-M}). PrP^{CJD-M} converted the 25K ³⁵S-hPrP-M and ³⁵S-hPrP-V lacking N-glycans to an ~18K proteinaseK-resistant form compatible with the proteinaseK-resistant core of PrP^{CJD} (Fig. 2, lanes 5, 6). Using predominantly glycosylated forms of ³⁵S-hPrP-sen, we saw that the PrP^{CJD} again preferentially generated the same 18K, presumably aglycosyl, conversion product, although some generation of the larger glycosylated conversion products was also observed (not shown). The ³⁵S-hPrP-M label was converted three-fold more efficiently than ³⁵S-hPrP-V by PrP^{CJD-M} (Fig. 2, lanes 5, 6; Fig. 4; $P = 0.0025$), similar to the findings from conversions with PrP^{BSE} (Figs 1 and 4).

The efficiency of cross-species conversion of hPrP-sen of either genotype by PrP^{BSE} was significantly less than the efficiency observed in either of the homologous conversion reactions: hPrP-M or hPrP-V with PrP^{CJD-M} ($P = 0.0005$ or 0.005 , respectively) or bovine (bo) PrP-sen with PrP^{BSE} ($P = 0.0005$) (Fig. 1, lanes 1–5, 8–10, 13, 14; Fig. 2, lanes 5–9; Fig. 4). On average, PrP^{BSE} converted boPrP-sen 30-fold more than hPrP-V ($P = 0.0005$), and 10-fold greater than hPrP-M ($P = 0.0005$). However, this low level of conversion was significantly higher than that seen on incubation of PrP^{BSE} with PrP-sen from hamsters ($P = 0.005$) or sheep homozygous for the AR genotype ($P = 0.025$)—hosts shown *in vivo* to be resistant to BSE—(Fig. 1, lanes 17, 20, 21). To put this into perspective, we evaluated the efficiency of conversion of hPrP with PrP^{Sc} from scrapie-infected sheep (ovPrP^{Sc}), a source of

agent of no measurable risk to the human population.

In conversions driven by ovPrP^{Sc(VQ)}, the conversion efficiency of ³⁵S-hPrP-M (Fig. 3, lane 1) ranked with that of mouse (lane 5) and sheep ov-AR (lane 4) proteins; no conversion of hamster (ha) PrP-sen was observed (lanes 6, 7). In contrast, much higher conversion efficiencies were observed with the ov-VQ and ov-AQ PrP-sen alleles. Thus, the conversion efficiencies of the different aglycosyl ovPrP-sen alleles by ovPrP^{Sc(VQ)} correlated with their sequence homology at codon 171: ov-VQ, ov-AQ $\gg$ ov-AR (Figs 3, 4)¹¹. These relative conversion efficiencies correlated with the fact that, although animals of VQ/VQ, AQ/VQ or AQ/AQ genotypes can develop natural scrapie (depending on breed) and can be experimentally induced to develop clinical scrapie (depending on agent strain) and BSE, animals with at least one copy of the AR allele seem to have enhanced survival following exposure to either natural or experimental disease¹⁶. Also, the lack of conversion of haPrP-sen by ovPrP^{Sc(VQ)} correlates with the lack of documented transmission of the sheep scrapie agent directly to hamsters (Fig. 4).

In summary, the most efficient conversion reactions were observed between homologous PrP-sen and PrP-res molecules, just as TSE transmissions are usually most efficient between hosts of homologous PrP genotype (Fig. 4). In contrast, little or no conversion was observed with non-homologous PrP combinations associated with a lack of transmission *in vivo*. Although overall conversion efficiencies varied and were influenced by the pretreatment and conversion GdnHCl concentrations, the optimal conversion conditions for each species fell within the range shown in Figs 1 and 2. Furthermore, variations of these conditions, the amount of PrP-res, or the glycosylation state of PrP-sen, did not seem to affect the relative efficiencies of conversion of the various PrP-sen molecules (Fig. 4). Comparison of the amino-acid sequences of human, sheep, bovine and other PrP molecules strongly implicated the loop structures of the protein¹⁹ as critical regions affecting the conversion of PrP-sen to PrP-res and for maintenance of at least part of the barrier to transmission of TSEs between species (see Supplementary Information).

Our results provide further evidence for a correlation between the efficiency of *in vitro* conversions and the transmissibilities of TSE

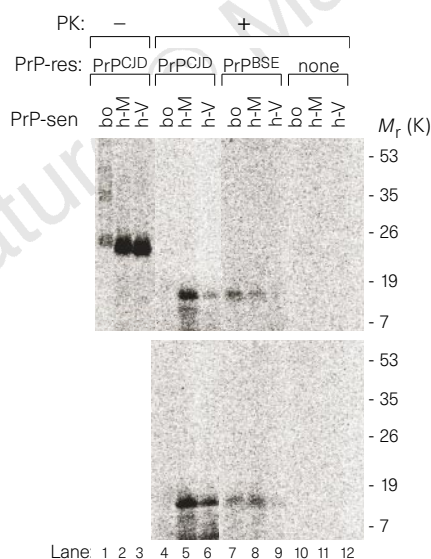


Figure 2 Comparison of cell-free conversions induced by PrP^{BSE} and PrP^{CJD}. Bovine (bo) and human (h-M, h-V) ³⁵S-PrP-sen proteins were labelled in the presence of tunicamycin (top panel, -PK, left) and incubated either alone (none) or with equivalent amounts (~0.5 µg) of PrP^{BSE} or human PrP^{CJD-M} before processing with or without PK treatment. The conversion reaction samples were split 1:10 between the -PK and +PK gel lanes. Phosphor autoradiographic images are shown of the labelled proteins from conversions using different concentrations of GdnHCl in the pretreatment and conversion phases of the reaction (see Methods): top panel, pretreatment in 2M GdnHCl followed by conversion in 1M GdnHCl; bottom panel, pretreatment in 2.5M GdnHCl, conversion in 1.5M GdnHCl.

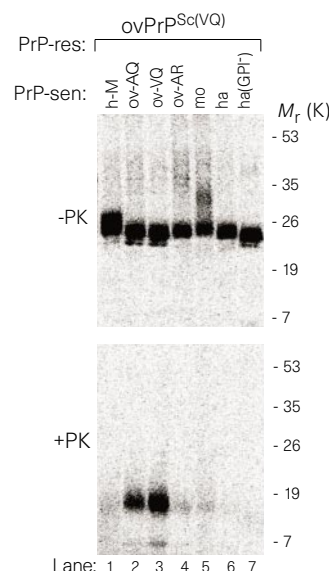


Figure 3 Cell-free conversions induced by sheep PrP^{Sc}. Human (h-M), mouse (mo), hamster (ha and ha-GPI⁻) and three allelic forms of sheep (ov-AQ, ov-VQ and ov-AR) ³⁵S-PrP-sen proteins were labelled in the presence of tunicamycin (-PK). These aglycosyl PrP-sen molecules were incubated with equivalent amounts (~0.5 µg) of ov-PrP^{Sc(VQ)} and processed as described in Methods. The reaction samples were split 1:10 (-PK: +PK) for analysis on the gels.

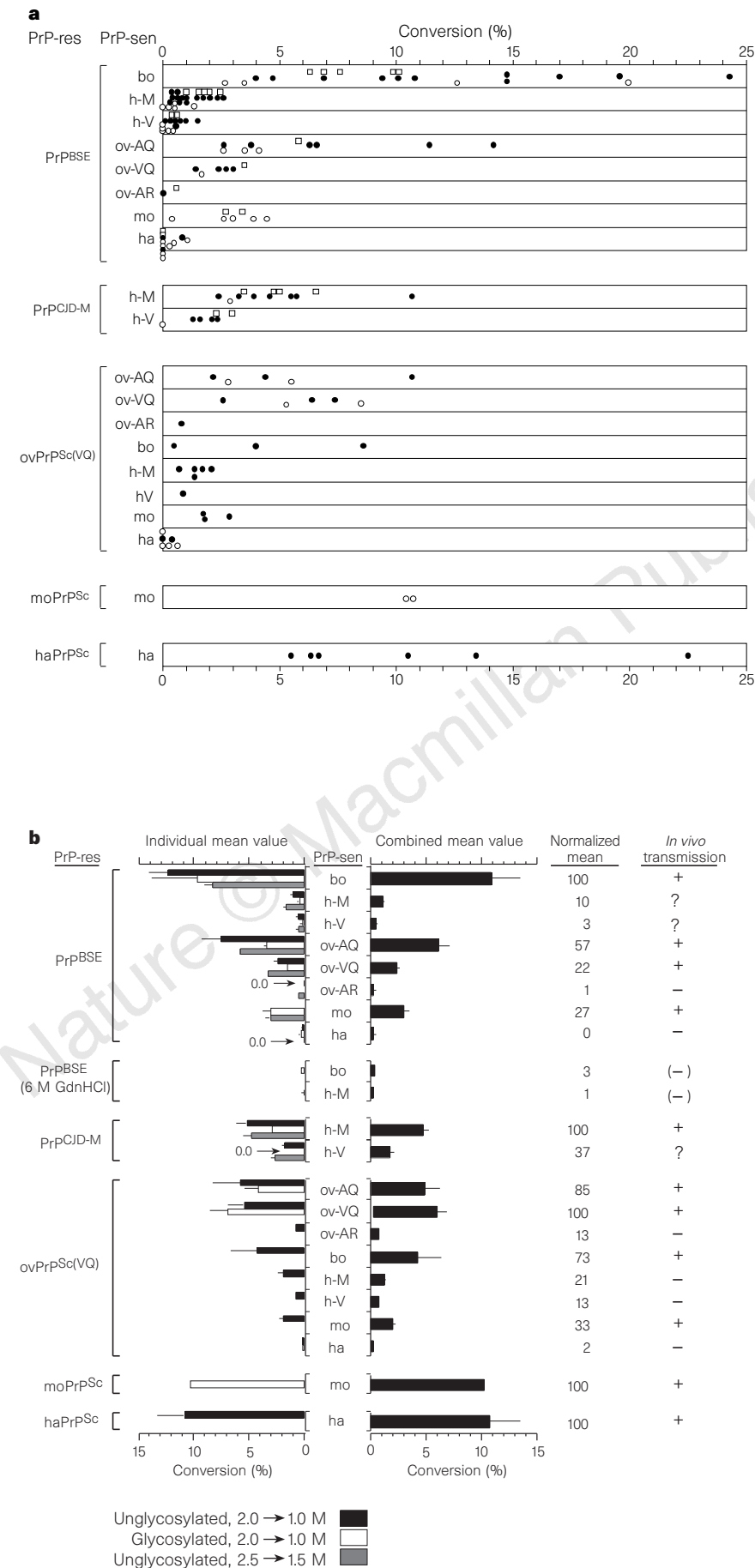


Figure 4 Percentages of ^{35}S -PrP-sen proteins converted to PK-resistant forms by incubation with PrP^{CJD}, PrP^{BSE}, and sheep, mouse or hamster PrP^{Sc}. **a**, Scatter graph of percentage conversion of PrP-sen to PK-resistant PrP forms in multiple, independent experiments. Each symbol represents percentage conversions observed in independent conversion reactions using the designated PrP-res incubated with glycosylated (open circles) or unglycosylated (filled circles) ^{35}S -PrP-sen with a pretreatment/conversion protocol of 2 M to 1 M GdnHCl or unglycosylated ^{35}S -PrP-sen with the 2.5 M to 1.5 M GdnHCl protocol (squares). **b**, Bar graph of percentage conversion of PrP-sen to PK-resistant PrP forms. Mean ($\pm$ s.e.m.) percentage conversion for each PrP-res/PrP-sen pair is shown calculated for the two GdnHCl pretreatment/conversion protocols and types of PrP-sen differentiated in **a** by the filled circles, open circles and squares (left) or summarized by combining the data from all experiments (right). The absence of a bar on the left means that no data were collected under those conditions except for the cases where the percentage conversions were negligible; such cases are shown by 0.0. As well as the results of **a**, this graph shows the negligible conversion produced using 6 M GdnHCl-pretreated PrP^{BSE}. Relative mean percentage conversions have been normalized against the mean of the homologous conversions induced by each type of PrP-res, with the latter set at 100%. Question marks acknowledge that it is likely, but not certain, that the human new variant CJD cases in hPrP-M129 homozygotes are due to BSE infections and that, although no new variant CJD cases have yet been observed in hPrP-V129 carriers, it is possible that all codon-129 genotypes may eventually be affected. Minus signs in brackets refer to the data indicating that 6 M GdnHCl treatment inactivates hamster scrapie infectivity²⁶, although this has not been demonstrated specifically for BSE infectivity.

diseases. It extends the basis for using the cell-free conversion reaction as an *in vitro* indicator of the transmission barrier to new species. We must emphasize that the cell-free assay is a test only of the molecular compatibility between PrP-res and PrP-sen of different sequences. Other factors such as the dose, strain and route of infection, the stability of the infectivity in the host, and the efficiency of its delivery to the central nervous system are all likely to be important *in vivo*. Nonetheless, some degree of molecular compatibility is likely to be essential in the transmission of TSE diseases, especially given the remarkable correlation between PrP conversion efficiencies and transmissibilities (Fig. 4). The conversion experiments with ^{35}S -hPrP-sen showed that the relative converting activity of PrP-res depends on its species of origin: $\text{PrP}^{\text{CJD-M}} > \text{PrP}^{\text{Sc}} > \text{PrP}^{\text{BSE}}$. The relatively low efficiency of conversion by PrP^{Sc} and PrP^{BSE} may explain at a molecular level the observed failure to transmit BSE to transgenic mice expressing human PrP²⁰ and the lack of an epidemiological link of CJD to sheep scrapie. It is premature to draw firm conclusions from our results about the likelihood of BSE passing to humans, although the results suggest that BSE would be no more inherently transmissible to humans than is sheep scrapie. □

Methods

Purification and analysis of PrP-res. PrP-res preparations were purified²¹ and stored at 4°C in 1%-sulphobetaine 3-14 in phosphate-buffered saline (PBS; 20 mM sodium phosphate and 120 mM NaCl, pH 7.4)¹⁷. Two or more independent isolates of each type of PrP-res were tested. Yields of PrP-res per g brain tissue were assayed by immunoblotting using R505, a polyclonal antiserum raised against a peptide epitope which, N-terminal amino acid apart, is common to all these species (sheep peptide 100-111; provided by J. Langeveld, Lelystadt, The Netherlands)²²; haPrP^{Sc} from Syrian golden hamsters infected with the 263K strain, ~50 µg g⁻¹; mouse (mo)PrP^{Sc} from VM/Dk mice infected with the 87V strain, ~10 µg g⁻¹; ovPrP^{Sc(VQ)} from natural scrapie cases in Cheviot sheep (provided by J. Foster, IAH-NPU, Edinburgh), ~1–5 µg g⁻¹; PrP^{BSE} from brainstem of BSE-affected cattle (provided by MAFF VI Centre, Thirsk), ~1 µg g⁻¹; PrP^{CJD} from cases of new variant or sporadic CJD (~1 µg g⁻¹).

Labelling, purification and analysis of ^{35}S -PrP-sen. ^{35}S -PrP-sen proteins were radiolabelled and purified using PrP-specific antisera as described¹⁷, except that they were eluted from the protein A-Sepharose beads using 0.1 M acetic acid at 22°C for 30 min and stored at 4°C. To prepare aglycosylated ^{35}S -PrP-sen, preincubation and labelling of cells were done in the presence of tunicamycin (2.5 µg ml⁻¹). Bovine adrenocortical cells (SBAC, ATCC RL-1796) were used to obtain radiolabelled boPrP-sen which was immunoprecipitated using a rabbit polyclonal PrP antiserum (R35) raised against a synthetic peptide sequence (residues 93–107) of bovine PrP (provided by R. Race, RML, Montana). Labelled hPrP-M and hPrP-V were produced from molecular clones expressed in human neuroblastoma cells and immunoprecipitated using the 3F4 monoclonal antibody^{23,24}. Endogenous moPrP-sen from mouse neuroblastoma (N2a) cells was collected using R30 serum. Recombinant mo-3F4 PrP-sen⁹ expressed in N2a cells was immunoprecipitated with the 3F4 antibody. ^{35}S -PrP-sen expressed for ovPrP clones encoding three of the sheep PrP genotypes¹¹ were expressed in N2a cells and immunoprecipitated with R521, a sheep-specific polyclonal antiserum against sheep PrP peptide 94–105 (provided by J. Langeveld²²). Hamster ^{35}S -PrP-sen proteins were immunoprecipitated from mouse fibroblast cells expressing recombinant haGPI⁹ PrP-sen or the full, unmodified haPrP-sen⁹.

Cell-free conversion reactions. To begin a conversion reaction²⁵, the PrP-res was sonicated, mixed with guanidine-HCl (GdnHCl) to a final concentration of 2.0, 2.5 or 6 M, typically in 8 µl and incubated at 37°C for 1–3 h. Approximately equal amounts of each ^{35}S -PrP-sen tested were lyophilized and reconstituted in conversion buffer (final composition in the conversion mix: 50 mM sodium citrate, pH 6.0, 5 mM cetyl pyridinium chloride, 1.25% (w/v) *N*-lauryl sarcosinate). Pre-treated PrP-res (in GdnHCl) and 8 M GdnHCl (to give a final concentration of 1 or 1.5 M) were added into the ^{35}S -PrP-sen solution (final volume, 16–24 µl) and incubated at 37°C for 3 days. The amount of different PrP-res proteins used in any single experiment was kept

constant at either ~0.5 or 0.8 µg. Using these amounts, the system was at or near saturation with PrP-res as the higher amount did not significantly increase the degree or efficiency of conversion (data not shown). After incubation, each reaction was split 1:10 and the major fraction digested with 100 µg ml⁻¹ proteinase K (PK) in Tris-saline (50 mM Tris-HCl, pH 8, 130 mM NaCl) for 1 h at 37°C. Pefabloc (2 mM) and thyroglobulin (20 µg) were added to each fraction (+ or -PK) and the proteins precipitated by methanol. Percentage conversions were determined by quantifying the proportion of radioactivity within a specific *M_r* range in each fraction (+PK and -PK) by phosphor autoradiographic imager analysis of SDS-PAGE gels. Aglycosylated ^{35}S -PrP-sen was quantified within an *M_r* range of ~24K to 26K; the glycoforms produced in non-tunicamycin-treated cells were scanned over an *M_r* of ~24K to 38K. PK-resistant products of conversions using aglycosylated ^{35}S -PrP-sen were quantified within an *M_r* range of ~18K to 20K; the variably glycosylated products of conversions using ^{35}S -PrP-sen labelled without tunicamycin were quantified over a *M_r* range of ~18K to 30K. The significance of differences in percentage conversion of PrP-sen/PrP-res pairs was calculated using the Mann-Whitney *U*-test.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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A minK-HERG complex regulates the cardiac potassium current I_{Kr}

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MinK is a widely expressed protein of relative molecular mass ~15K that forms potassium channels by aggregation with other membrane proteins¹⁻³. MinK governs ion channel activation⁴, regulation by second messengers^{5,6}, and the function and structure of the ion conduction pathway^{7,8}. Association of minK with a channel protein known as KvLQT1 produces a voltage-gated outward K^+ current (I_{Ks}) resembling the slow cardiac repolarization current (I_{Kr})^{9,10}. HERG, a human homologue of the *ether-a-go-go* gene of the fruitfly *Drosophila melanogaster*, encodes a protein that produces the rapidly activating cardiac delayed rectifier (I_{Kr})^{11,12}. These two potassium currents, I_{Ks} and I_{Kr} , provide the principal repolarizing currents in cardiac myocytes for the termination of action potentials^{13,14}. Although heterologously expressed HERG channels are largely indistinguishable from native cardiac I_{Kr} , a role for minK in this current is suggested by the diminished I_{Kr} in an atrial tumour line subjected to minK antisense suppression¹⁵. Here we show that HERG and minK form a stable complex, and that this heteromultimerization regulates I_{Kr} activity. MinK, through the formation of heteromeric channel complexes, is thus central to the control of the heart rate and rhythm.

We studied a clonal line of Chinese hamster ovary (CHO) cells stably expressing HERG (CHO-HERG) by whole-cell voltage clamp. CHO-HERG cells showed voltage-gated K^+ currents typical of human I_{Kr} which were absent from untransfected CHO cells (Fig. 1a). HERG currents showed voltage-dependent activation and deactivation, inward rectification at positive potentials, and sensitivity to E4031 like that of endogenous cardiac I_{Kr} (refs 13, 14) and HERG- K^+ channels expressed in *Xenopus* oocytes^{11,12}. CHO cells transfected with minK alone gave no voltage-gated currents (Fig. 1b; $n = 6$), as already reported¹⁶. We confirmed the activity of minK by co-expression with KvLQT1, which produced I_{Ks} -like currents (data not shown) as expected^{9,10}. When minK was co-expressed in CHO-HERG cells, an I_{Kr} -like current similar to HERG, but larger, was seen (Fig. 1c).

HERG and HERG-minK current densities were quantified by measuring maximal tail currents at -40 mV after a 2-s depolarizing test pulse to +60 mV. Currents were normalized for plasma membrane surface area by cell capacitance. Co-expression of minK and HERG resulted in a doubling of the K^+ current density (HERG, 12.8 ± 1.6 pA per pF, $n = 55$; HERG-minK, 24.0 ± 3.45 pA per pF, $n = 39$; $P < 0.001$) (Fig. 2a). To assess the specificity of this response, the effect of D77N-minK on HERG currents was studied. D77N-minK is expressed on the surface of *Xenopus* oocytes as is wild type, but it produces no I_{Ks} current². When D77N-minK was co-expressed with HERG, there was no increase in K^+ current (tail current density, 12.9 ± 1.7 pA per pF, $n = 37$; P , NS).

Whole-cell current is described by the equation $I = n(P_o)i$, where I is the measured current, n the number of functional channels, P_o

the probability of channel opening, and i the unitary channel current amplitude. Thus, an increase in current density must be due to changes in channel gating kinetics (P_o), i , n , or to changes in membrane surface area. First, the gating kinetics of HERG and HERG-minK channels were compared. MinK produced a hyperpolarizing shift of 6–10 mV in the voltage dependence of activation (Fig. 2b). The current-voltage relationship (Fig. 2c) showed a hyperpolarizing shift of similar magnitude at voltages more negative than +20 mV and at more positive potentials, up to an 18-mV shift in negative slope conductance as a result of increased steady-state inactivation (Fig. 2d). These results indicated that minK produced an increase in the voltage sensitivity of activation and inactivation at intermediate voltages. These changes in gating could not, however, account for increased maximal current densities. No significant differences were measured for time constants of activation at +60 mV or deactivation at -40 mV between HERG and HERG-minK channels (τ_{act} : HERG, 282 ± 72 ms, $n = 16$; HERG-minK, 246 ± 38 ms, $n = 17$, P is NS; τ_{fast} deactivation: HERG, 477 ± 78 ms, $n = 16$; HERG-minK, 428 ± 64 ms, $n = 17$, P is NS).

As changes in gating kinetics did not explain the increased current density, single channel conductances were determined. Unitary conductances for HERG and HERG-minK channels between -30 and +40 mV were 10.3 ± 0.5 pS ($n = 8$) and 10.6 ± 0.4 pS ($n = 14$), respectively (Fig. 3). Thus, changes in i also failed to account for increased whole-cell current density. Neither did changes in membrane area lead to increased currents; the surface areas of cells expressing HERG or HERG-minK were not significantly different (34.1 ± 2.7 pF, $n = 50$ and 29.7 ± 2.7 pF, $n = 46$, respectively). Therefore, a change in the number of functional channels in the plasma membrane must underlie the effect of minK on HERG currents. These currents appear to result from HERG-minK channels rather than induction of non-HERG K^+ conductances because E4031, a specific blocker of I_{Kr} , abolished all voltage-gated K^+ currents in HERG- and HERG-minK-expressing cells (Fig. 1d) at concentrations used to distinguish between I_{Kr} and I_{Ks} (0.5 and 1.0 μ M)¹³.

To assess whether minK influenced HERG by direct physical contact and formation of a stable heteromeric complex, the proteins were epitope-tagged for protein analysis. A c-Myc epitope at the

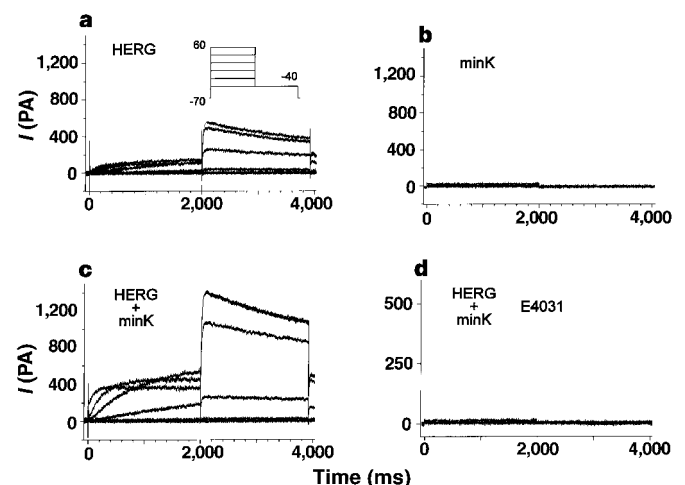


Figure 1 Functional expression of HERG and HERG with minK. **a**, A CHO-HERG cell showing voltage-gated K^+ current typical for I_{Kr} . The holding potential was -70 mV, cells were depolarized to potentials between 0 and +60 mV and tail currents measured during repolarization to -40 mV. **b**, A CHO cell transiently transfected with minK showing no voltage-gated currents. **c**, A CHO-HERG cell transiently transfected with minK showing HERG-minK, I_{Kr} -like K^+ currents. **d**, E4031 at 500 nM completely blocks all voltage-activated K^+ conductance in a cell expressing minK and HERG.

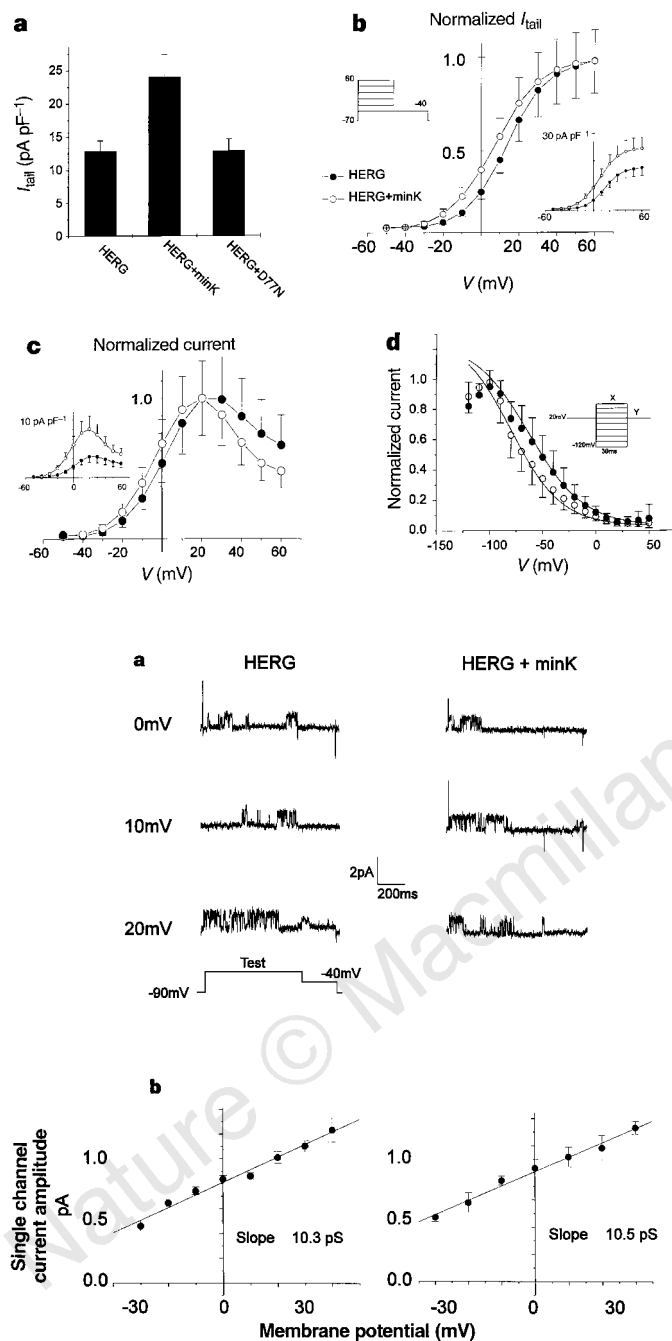


Figure 3 Single-channel currents from HERG and HERG-minK. **a**, Cell-attached patch recordings of single K^+ channel currents from CHO-HERG cells. Voltage protocol is shown below the current traces. **b**, Single-channel current-voltage curves showing unitary conductances for HERG and HERG-minK.

carboxy terminus of HERG had no measurable effect on channel currents (data not shown). Similarly, a haemagglutinin (HA) epitope between minK amino acids 22 and 23 was also well tolerated, as shown before⁵, and did not alter its augmentation of HERG currents or formation of functional I_{Ks} channels with KvLQT1 (data not shown). Expression of HA-minK produced three specific bands corresponding to relative molecular masses (M_r) of $\sim 15K$ – $25K$, which represent unmodified and glycosylated forms of minK (Fig. 4a), as expected⁴. Expression of HERG-Myc yielded a single band of $M_r \sim 130K$, consistent with the predicted mobility of HERG (Fig. 4a).

When HERG-Myc and HA-minK were co-expressed, immuno-

Figure 2 MinK alters functional expression of HERG in CHO cells. **a**, HERG current density is augmented by minK but not D77N-minK. The bar graph summarizes current densities for CHO-HERG cells ($n = 55$ cells), CHO-HERG cells transfected with wild-type minK ($n = 39$ cells), or CHO-HERG cells transfected with D77N-minK ($n = 37$ cells). Peak tail currents were measured as for Fig. 1 and normalized for cell capacitance. **b**, Voltage-activation curves; normalized peak tail current density at -40 mV was plotted against test potential, demonstrating a left shift in voltage-dependent activation. Current density is greater for HERG-minK at all potentials that activate current (inset: data plotted without normalization). Data were fitted to the Boltzmann relation, $y = 1/(1 + \exp[(V_h - V)/k])$, where V is the test potential, V_h the potential at half-maximal activation ($+12$ mV for HERG and $+6$ mV for HERG-minK) and k is the slope factor (11 mV for both). **c**, Current-voltage relation for HERG and HERG-minK cells. Normalized current during depolarization is plotted against the membrane potential. There is a hyperpolarizing shift in voltage dependence that is most pronounced at potentials greater than $+20$ mV. Current density is greater for HERG-minK than HERG alone at all voltages (inset: data plotted without normalization). **d**, Steady-state inactivation³⁰ was assessed by brief steps to various hyperpolarized potentials from $+20$ mV; the plot shows peak current released from inactivation at $+20$ mV against test potential. Inset shows voltage pulse protocol with X showing the voltage steps and Y indicating where current was measured.

precipitation of either protein led to recovery of both channel subunits (Fig. 4b,c; $n = 3$). Although D77N-minK did not augment HERG currents (Fig. 2a), HA-D77N-minK did associate with HERG to form a stable complex like the wild-type protein (Fig. 4c, lane 4; $n = 2$). The association between HERG and minK was specific; when Myc-tagged connexin 43 (Cx43-Myc), an unrelated membrane channel subunit, was co-expressed with both HA-minK and HERG-Myc, immunoprecipitation with anti-HA antibody recovered only HERG and no Cx43 (Fig. 4d).

Although co-expression of HERG and minK increased the number of functional channels, it did not alter the amount of HERG protein on the cell surface. Western blot analysis was performed on membrane and cytosolic/microsomal fractions prepared from CHO cells transiently transfected with HERG-Myc and Cx43-Myc, either alone or in combination with HA-minK or HA-D77N-minK. In each case, all detectable HERG-Myc was recovered in the membrane fraction and the abundance of HERG-Myc (and Cx43-Myc) was unaffected by co-expression of HA-minK or HA-D77N-minK (Fig. 4e; $n = 3$). The same results were obtained when either HA-minK or HA-D77N-minK was expressed in the stable CHO-HERG cell line; neither the membrane localization of HERG nor its level of expression was affected (Fig. 4f). Co-expression of minK with HERG also did not protect HERG from degradation. Pulse-chase analysis showed time constants of degradation of ~ 11.2 h and 11.6 h for HERG in the absence and presence of minK, respectively (Fig. 4g; $n = 2$).

Our results show that minK physically associates with HERG and that the interaction leads to increased I_{Ks} current density. The fact that both non-glycosylated and glycosylated forms of minK bind to HERG suggests that association takes place early in the protein synthesis and trafficking pathway, probably in the endoplasmic reticulum. As minK increases the number of functional HERG channels in the plasma membrane but does not alter cell-surface expression of the HERG protein, minK appears to increase the active fraction in a membrane pool containing active and dormant channels. This type of shift in channel activity may underlie upregulation of minK function in oocytes after treatment with cyclic AMP¹. Similarly, cAMP has been shown to regulate the proportion of functional nicotinic acetylcholine receptor channels in chick ciliary ganglion cells¹⁷. Such mode switching is reminiscent of the gating behaviour seen in RCK4 K^+ channels, where a fraction of channels in oocyte patches fail to open with depolarization when external K^+ is removed¹⁸.

The consequences of minK interaction with KvLQT1 and HERG

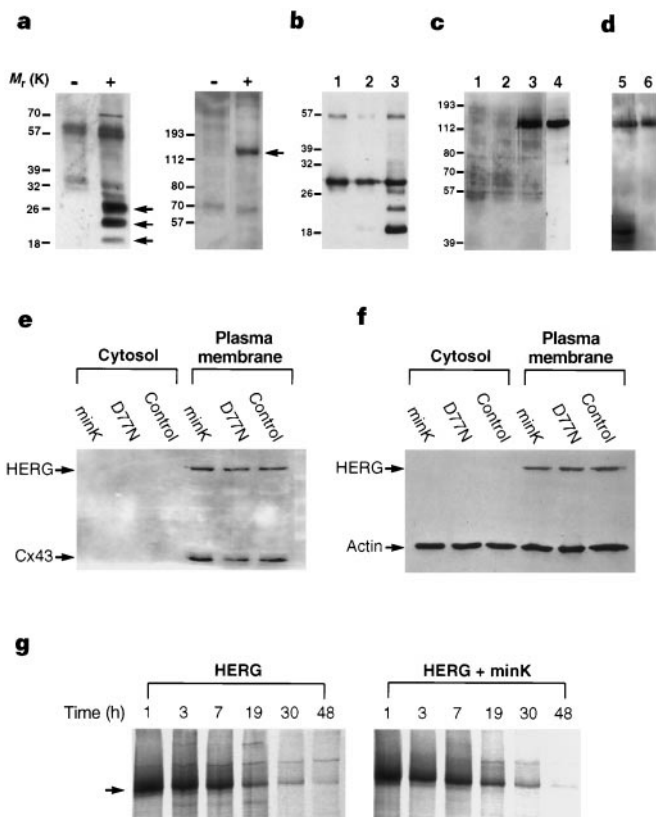


Figure 4 Association of minK and HERG. **a**, Expression of epitope-tagged constructs. Cell lysates probed with anti-HA, showing three bands corresponding to native and glycosylated forms of minK (left), or with anti-Myc, showing a single band of ~130K as predicted for HERG protein (right). Control lanes (-) are lysates from untransfected cells. **b**, Co-immunoprecipitation of minK with HERG. Lysates were immunoprecipitated with anti-Myc antibody followed by SDS-PAGE and detection with anti-HA. Lanes: 1, untransfected cells; 2, cells transfected with HERG-Myc; 3, cells transfected with HERG-Myc and HA-minK. **c**, Co-immunoprecipitation of HERG with minK or D77N-minK. Lysates were immunoprecipitated with anti-HA antibody followed by SDS-PAGE and detection with anti-Myc antibody. Lanes: 1, untransfected cells; 2, cells transfected with HA-minK; 3, cells transfected with HA-minK and HERG-Myc; 4, cells transfected with HA-D77N-minK and HERG-Myc. **d**, Specificity of HERG-minK interaction. Lane 1, lysate from cells transfected with HA-minK, HERG-Myc, and Cx43-myc probed with anti-cmyc monoclonal antibody capable of detecting both HERG-Myc and Cx43-myc. Lane 2, same lysate immunoprecipitated with anti-HA antibody, followed by SDS-PAGE and detection with peroxidase-conjugated anti-Myc antibody, showing recovery of only HERG-Myc. **e**, Epitope-tagged HERG localizes to the plasma membrane. CHO cells were transfected with HERG-Myc either alone (control) or in combination with HA-minK or HA-D77N-minK. Cx43-Myc was included in all transfections. Lysates were fractionated into plasma cytosolic and membrane fractions. HERG-Myc and Cx43-Myc were recovered exclusively in the membrane fraction. Co-expression of minK or D77N-minK did not affect the abundance of HERG ($n = 3$). **f**, CHO-HERG cells stably expressing wild-type HERG were fractionated as described and detected with antisera against HERG. Unlike actin, HERG localized exclusively in the membrane fraction. Co-expression of HA-minK or HA-D77N-minK did not affect the abundance of HERG ($n = 3$). **g**, Pulse-chase analysis. The rate of degradation of metabolically labelled HERG was not significantly different in cells transfected with HERG-Myc alone or in combination with minK.

are surprisingly similar. We have shown here that minK alters HERG channel gating kinetics and increases macroscopic I_{Kr} current density. By an as-yet unknown mechanism, minK also acts on KvLQT1 to alter its gating kinetics (to those characteristic of I_{Ks}) and to increase macroscopic current^{9,10}. Although HERG produces I_{Kr} -like currents when expressed alone whereas KvLQT1 strictly requires minK to create recognizable I_{Ks} channels, this may be a quantitative rather than a qualitative difference. In this respect, it is notable that D77N-minK fails to augment I_{Kr} currents even though it physically associates with HERG protein (Figs 2a, 4c), just as it fails to produce I_{Ks} currents but can compete with wild-type minK when co-expressed in *Xenopus* oocytes². This suggests that similar molecular features of minK are important for influencing the function of HERG and KvLQT1.

Inasmuch as minK is required for I_{Ks} and alters I_{Kr} , its expression and modulation by second messengers^{5,6} will play a central role in determining the amount and character of the K^+ conductances in individual cardiac myocytes. Moreover, expression of minK, HERG, KvLQT1 and other K^+ channel proteins is region-specific in the heart and varies in pathological states, producing spatial heterogeneity in current densities and phenotypes^{19,20}. Mutations in KvLQT1 and HERG produce hereditary long-QT syndrome (LQTS) by decreasing I_{Ks} and I_{Kr} (refs 21, 22), so minK should be expected to act as a modifier of genetically determined LQTS. As HERG has also been implicated in acquired arrhythmias^{11,23}, minK may be involved in susceptibility to acquired LQTS and pro-arrhythmic drug phenomena. In the mouse, disruption of the *minK* gene results in *shaker/waltzer* behaviour and hair-cell degeneration²⁴. This null phenotype suggests an association between mutations in minK and Jervell and Lange-Nielsen syndrome, an autosomal recessive form of LQTS that is associated with deafness²³.

These results indicate a broad role for minK in determining the functional expression of delayed rectifier K^+ currents in the heart. The physical and functional interaction of minK with two diverse K^+

channel types (KvLQT1 and HERG) and its wide tissue distribution (heart, ear, kidney, pancreas, T cells and gastrointestinal tract)^{25–28} suggest that the role of minK in normal physiology and disease pathogenesis may be even more extensive. □

Methods

Plasmid construction and transfections. HERG was epitope-tagged by replacing the three C-terminal amino acids of the wild-type protein with ISMEQKLISEEDLN, derived from the *c-myc* gene recognized by monoclonal antibody 9E10. Construction of HA-tagged minK and D77N-minK have been described¹². CHO cells stably expressing HERG were obtained by transfection with a pCI-based vector carrying HERG and selection with G418. Transient transfections were carried out by electroporation with the indicated minK constructs together with green fluorescent protein.

Electrophysiology. Cells were studied on an inverted microscope equipped with epifluorescence optics for GFP detection and electrophysiology experiments. Whole-cell voltage clamp was done with an internal solution of KCl 120 mM, MgCl₂ 2 mM, CaCl₂ 0.5 mM, EGTA 5 mM, ATP-Mg₄ 4 mM, HEPES 10 mM (pH 7.2; osmolality, 280 ± 10 mOsm). External bath solution consisted of NaCl 150 mM, CaCl₂ 1.8 mM, KCl 4 mM, MgCl₂ 1 mM, glucose 5 mM, HEPES 10 mM (pH 7.4; osmolality, 320 ± 10 mOsm). Experiments were done at room temperature. On-cell and excised patch configuration was used to study single HERG channels. Cells were bathed in KCl 120 mM, NaCl 10 mM, MgCl₂ 2 mM, HEPES 10 mM (pH 7.4), glucose 5 mM. Signals were analogue-filtered at 2,000 Hz and sampled at 5–10 kHz. Amplitude histograms at each membrane potential were constructed to obtain single-channel conductance. Data were analysed using electrophysiology software (pClamp, Axon Instruments).

Protein analysis. Transfected cells were lysed in Tris lysis buffer (0.15 M NaCl, 1% NP-40, 1% CHAPS, 20 mM NaF, 10 mM Na₃VO₄, 1 mM PMSF and 50 mM Tris, pH 7.4). Lysates were cleared by centrifugation at 10,000g for 30 s. For direct immunoblot analysis, samples were separated by SDS-PAGE and epitope-tagged proteins detected with appropriate monoclonal antibodies conjugated to horseradish peroxidase (Boehringer Mannheim). For detection of untagged HERG, membranes were incubated with rabbit anti-HERG

polyclonal antisera²⁹, followed by horseradish peroxidase-conjugated protein A. Lysates were immunoprecipitated with either 5 µg anti-HA 12CA5 or 3 µg anti-Myc 9E10 monoclonal antibody for 2 h at 4 °C, followed by incubation with protein G–Sepharose for 1 h at 4 °C. For pulse-chase analysis, cells were labelled for 30 min with ³⁵S-methionine/cysteine, chased in medium with excess unlabelled methionine/cysteine for varying times and collected by detergent lysis. After immunoprecipitation and SDS–PAGE, gels were visualized by fluorography and quantified by densitometry (Molecular Analysis, Biorad). For subcellular fractionation, adherent cells were detached by incubation with EDTA and gently disrupted by Dounce homogenization. Cellular lysates were layered on 45% sucrose cushions and membrane and cytosolic/microsomal fractions recovered after centrifugation at 7,000g for 20 min.

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P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1

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The factors determining disease severity in malaria are complex and include host polymorphisms, acquired immunity and parasite virulence¹. Studies in Africa have shown that severe malaria is associated with the ability of erythrocytes infected with the parasite *Plasmodium falciparum* to bind uninfected erythrocytes and form rosettes^{2–5}. The molecular basis of rosetting is not well understood, although a group of low-molecular-mass proteins called rosetins have been described as potential parasite ligands⁶. Infected erythrocytes also bind to endothelial cells, and this interaction is mediated by the parasite-derived variant erythrocyte membrane protein PfEMP1 (refs 7, 8), which is encoded by the *var* gene family^{9–11}. Here we report that the parasite ligand for rosetting in a *P. falciparum* clone is PfEMP1, encoded by a specific *var* gene. We also report that complement-receptor 1 (CR1) on erythrocytes plays a role in the formation of rosettes and that erythrocytes with a common African CR1 polymorphism (SI(a)⁺)¹² have reduced adhesion to the domain of PfEMP1 that binds normal erythrocytes. Thus we describe a new adhesive function for PfEMP1 and raise the possibility that CR1 polymorphisms in Africans that influence the interaction between erythrocytes and PfEMP1 may protect against severe malaria.

We studied the role of PfEMP1 in rosetting using the clone R29 of *P. falciparum*, which was divided into isogenic rosetting (R29R⁺) and non-rosetting (R29R[−]) subpopulations by sedimentation in gelatin¹³ (see Methods). Each parasite genome is estimated to contain 50–150 *var* genes¹¹, and switching of expression from one *var* to another gives rise to antigenic variation¹⁴. To study the *var* genes expressed by R29R⁺ and R29R[−], we used the polymerase chain reaction with reverse transcription (RT-PCR) and degenerate oligonucleotide primers to the first cysteine-rich Duffy-binding-like domain (DBL-1) of the *var* genes. This DBL-1 domain of known *var* genes has regions of very high sequence homology interspersed with regions of variable sequence¹¹. The RT-PCR product from R29R⁺ RNA consisted of a single major band, whereas three distinct bands were visible from R29R[−] RNA (Fig. 1a). RT-PCR products were cloned into a pCRII vector and those with inserts of the appropriate size for DBL-1 (400–600 base pairs (bp)) were sequenced. From the cloning of the R29R⁺ RT-PCR product, 13 recombinant plasmids contained inserts of 400–600 base pairs. These all contained the DBL-1 sequence (called R29R⁺*var*1; Fig. 1b), which is identical to the *var* sequence described for the R29 clone¹⁰. From the cloning of the R29R[−] RT-PCR product, five different DBL-1 sequences were detected, namely R29R[−]*var*2 to R29R[−]*var*6 (Fig. 1b). None of the recombinant plasmids from the R29R[−] RT-PCR product contained the R29R⁺*var* sequence found in rosetting parasites. Using primers specific for R29R⁺*var*1, we confirmed by RT-PCR that this gene is specific to the R29R⁺ population and is not expressed by the R29R[−]

non-rosetting parasites (Fig. 1c). The presence of amplifiable RNA in the R29R⁻ sample was demonstrated by RT-PCR with specific primers to *R29R⁻var3* which gave a 550-bp band with R29R⁻ RNA (Fig. 1c). Thus, a particular *var* gene, *R29R⁺var1*, is expressed by rosetting parasites but not by non-rosetting parasites of the R29 clone. This finding is consistent with a role for *var*/PfEMP1 in rosetting.

To examine the functional properties of *R29R⁺var1*, the sequence was extended by PCR-walking using vectorette libraries¹⁵. We obtained seven overlapping clones encoding all of exon 1 (the entire extracellular domain and the transmembrane region), the intron, and the 5' half of exon 2 (the acidic intracellular domain). The clones were sequenced in both directions and gave an open reading frame of 8.2 kilobases (kb). *R29R⁺var1* has the characteristic *var* gene structure^{9,11}, with the extracellular portion consisting of four DBL domains and a cysteine-rich interdomain region (CIDR) (Fig. 1d).

To study the binding properties of *R29R⁺var1* to red blood cells (RBCs), constructs were made to express individual DBL domains and the CIDR in COS-7 cells as chimeric proteins with the herpes simplex virus glycoprotein D¹⁶. COS-7 cells were transiently transfected, and surface expression of DBL domains was determined by immunofluorescence assay with monoclonal antibodies against the herpes simplex glycoprotein D¹⁷. Each domain of the *R29R⁺var1* gene was expressed on the surface of COS-7 cells except DBL-4 (Fig. 1d). The transfected COS-7 cells were tested for their ability to bind RBC. DBL-1 of *R29R⁺var1* bound RBC, whereas DBL-2, DBL-3, and CIDR did not (Fig. 1d, e). As evidence that not all DBL-1 domains bind RBC, DBL-1 expressed by a non-rosetting parasite (A4 (ref. 10); gift from J. Smith) was expressed on ~2% of COS-7 cells but did not bind RBC.

We studied the effect of the anti-CD36 monoclonal antibody OKM5 on RBC binding to DBL-1 of *R29R⁺var1* because PfEMP1 is known to bind to CD36 (refs 7, 8) and previous work has shown that CD36 is expressed at low levels on RBC and can rarely act as a rosetting receptor¹⁸. OKM5 (100 µg ml⁻¹) did not inhibit RBC binding to DBL-1 of *R29R⁺var1* expressed in COS-7 cells (36 positive cells with OKM5, compared with 32 positive cells with an isotype-matched control; mean binding in 50 fields at 200× magnification from two experiments). R29 rosetting also is not inhibited by OKM5 (ref. 19), so CD36 binding does not play a role in the interaction between PfEMP1 and RBC described here.

Confirmation that binding of RBC to DBL-1 is the basis of

Table 1 Binding of CR1-deficient RBC to DBL-1 of *R29R⁺var1* expressed in COS-7 cells

Donor	RBC binding*
CR1-deficient (frozen)	0
Control	27
CR1-deficient (M.H.)	0
Control	45
CR1-deficient (B.S.)	7
Control	22

* Number of positive COS-7 cells in 50 fields at 200× magnification. Data shown represent the mean binding from two independent experiments for each sample.

rosetting was hampered by the fact that the RBC receptor for R29 was unknown. We therefore tested 23 RBC variants that were negative or null for a range of high-frequency blood group antigens to determine whether any of these RBC were unable to form rosettes with five *P. falciparum* clones/lines. Initial experiments were with frozen RBC, and the experiments were repeated with fresh RBC for any variant that showed reduced rosetting. The Knops null RBC was the only one to show consistently reduced rosetting (Fig. 2a). Knops null RBC express low copy numbers of complement-receptor 1, having fewer than 100 CR1 molecules per RBC compared with the normal range of 100 to 800 (refs 20, 21). Previous work had shown that the ABO blood grouping influences the size of rosettes formed but not the overall rosette frequency²². Our results, however, were not dependent on the ABO blood group of the RBC. To exclude any artefact resulting from the purification of infected RBC for the rosette-reformation assay, fresh CR1-deficient RBC were obtained from another donor (B.S.; CR1 copy number 60), and the rosetting R29R⁺ parasites were cultured *in vitro* for 9 days in the CR1-deficient and control normal RBC. Blinded assessments of rosette frequency were made every cycle, and the reduced rosetting of CR1-deficient RBC was confirmed (Fig. 2b). The parasitaemia in CR1-deficient RBC was equivalent to the control, and Giemsa-stained smears showed that the parasites were morphologically normal. We have therefore demonstrated reduced rosetting of CR1-deficient RBC from four different donors (two frozen and two fresh) either by rosette reformation assay or by continuous culture.

Our results suggest that CR1 plays a role in the formation of rosettes. We therefore examined the effect of soluble recombinant CR1 (sCR1; ref. 23) on rosetting. sCR1 inhibited rosette formation in three out of four parasite clones/lines tested (Fig. 2c, d). These

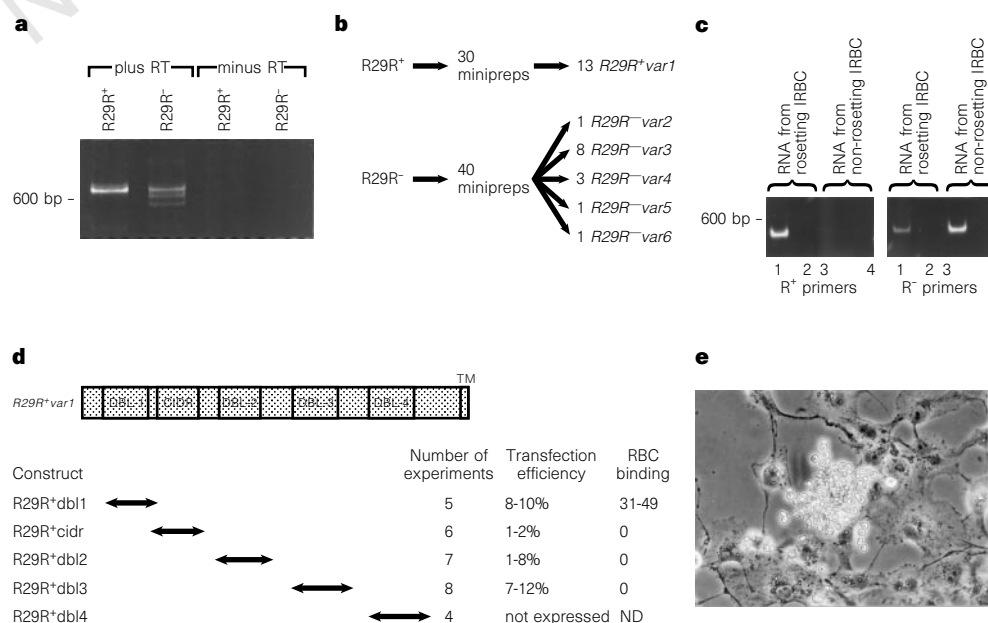


Figure 1 *Var* gene expression and RBC binding in *P. falciparum* clone R29. **a**, RT-PCR analysis of *var* gene expression in rosetting (R29R⁺) and non-rosetting (R29R⁻). To control for possible genomic DNA contamination, reactions with and without reverse transcriptase (RT) were performed in parallel. **b**, Cloning and sequence of RT-PCR products from **a**. All minipreps with inserts of 400–600 bp were sequenced. **c**, RT-PCR analysis with specific primers to *R29R⁺var1* (R⁺ primers) and *R29R⁻var3* (R⁻ primers). IRBC, infected RBC. **d**, Expression constructs and RBC binding of domains of *R29R⁺var1*. ND, not determined. **e**, RBC binding to COS-7 cell transiently transfected with DBL-1 of *R29R⁺var1*.

results are consistent with the importance of CR1 in the formation of rosettes. The reason for the failure of sCR1 to inhibit rosetting by PAR⁺ is unknown, although it may relate to additional receptors or affinity of PAR⁺ for sCR1.

CR1-deficient RBC were used to determine the specificity of RBC binding to DBL-1 of *R29R⁺var1*. CR1-deficient RBC from three donors showed reduced or absent binding to DBL-1 compared with control RBC (Table 1). Thus, a particular *var* gene (*R29R⁺var1*) is expressed by rosetting but not by non-rosetting parasites of the R29 clone, and DBL-1 of this *var* gene binds RBC with the same specificity as R29 rosetting parasites. Together, these data provide strong evidence that PfEMP1 encoded by *R29R⁺var1* is the rosetting-mediating ligand in R29.

The high mortality of *P. falciparum* in endemic areas has resulted in the selection for polymorphisms that afford protection from severe malaria^{24,25}. The association between the rosetting phenotype and severe disease from *P. falciparum* suggests that natural RBC variants that support rosetting less well may have been selected to a high frequency by reducing mortality from malaria. The SI(a⁻) CR1

blood-group polymorphism occurs in ~30% of African Americans but is rare in Caucasians¹². We tested a blinded panel of SI(a⁺) and SI(a⁻) RBC for their ability to bind to DBL-1 of *R29R⁺var1* expressed in COS-7 cells. RBC from each donor were characterized for their CR1 copy number and SI(a) type (Table 2). SI(a⁻) RBC consistently showed lower binding to DBL-1 than did SI(a⁺) controls ($P = 0.0001$). Confirmation that SI(a⁻) and another high-frequency polymorphism of CR1 in Africans (McC(b⁺))¹² are protective alleles will require molecular definition of the polymorphisms and their evaluation in large case-control studies of severe malaria.

Rosetting is a heterogeneous phenomenon in which a number of red-cell ligands (ABO blood group²², CD36 (ref. 18)) and soluble molecules (immunoglobulins²⁶, sulphated glycoconjugates¹⁹) differentially modify the degree of rosette formation in different isolates. Small proteins (rosetteins) on the infected RBC surface have also been associated with the rosetting phenotype⁶. Multiple pathways of rosette formation may exist, but the fact that rosetting of five independent parasites is reduced in RBC with low expression of CR1 suggests that CR1 may play a common but not exclusive role

Table 2 Binding of a blinded panel of SI(a⁺) and SI(a⁻) erythrocytes to COS-7 cells expressing DBL-1 of *R29R⁺var1*

Binding† to COS-7 cells expressing DBL-1								
Donor	Race*	CR1 copy number	SI(a) Type	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Set 1								
960673	W	180	—	7, 8	21, 15	ND‡	ND	ND
960675	B	251	—	13, 9	14, 7	ND	ND	ND
960676	B	309	—	31, 23	38, 31	ND	ND	ND
960671	W	229	+	38, 29	56, 32	ND	ND	ND
960670	B	205	+	61, 55	72, 81	ND	ND	ND
960674	W	372	+	62, 61	76, 70	ND	ND	ND
Set 2								
960729	B	273	—	ND	19, 14	21, 36	ND	ND
960014	B	298	+	ND	47, 38	49, 66	ND	ND
920560	B	332	+	ND	70, 47	85, 87	ND	ND
960048	B	336	+	ND	71, 87	76, 123	ND	ND
Set 3								
960746	B	274	—	ND	ND	ND	0, 8, 2	11, 19, 8
960745	B	342	—	ND	ND	ND	9, 14, 7	18, 28, 11
960748	H	325	+	ND	ND	ND	53, 38, 38	53, 88, 68

* W, white; B, black; H, hispanic

† Data shown are number of positive COS-7 cells in 50 fields at 200× magnification for two coverslips (experiments 1–3) or three coverslips (experiments 4 and 5). The binding data were subjected to a three-way analysis of variance (SI(a) type, experiment, donor; PROC GLM procedure, SAS Institute). The difference between respective mean binding values (SI(a⁺) 15.8 ± 1.9 s.e. versus SI(a⁻) 2.3 ± 3.3 s.e.) accounted for two-thirds of the overall observed variability and was significant at the 0.0001 level (18.3, 42 degrees of freedom).

‡ ND, not determined.

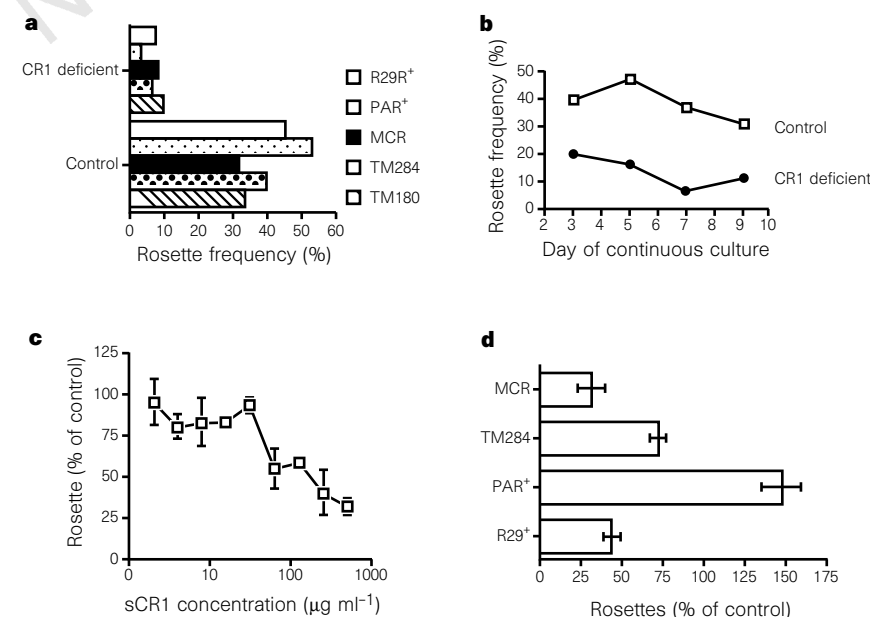


Figure 2 CR1 is required for *P. falciparum* rosetting. **a**, Rosette formation of fresh CR1-deficient RBC (donor MH; CR1 copy number, 28) with five parasite clones/lines. **b**, Rosetting of R29 grown in continuous culture in CR1-deficient (donor BS; CR1 copy number, 60) and normal RBC. **c**, Dose-dependent inhibition of R29 rosetting by sCR1. Rosetting at each sCR1 concentration is expressed as a percentage of the rosette frequency in control R29 with no added sCR1. Data shown are mean and standard error of triplicate determinations of rosette frequency at each concentration from a representative experiment. Two additional experiments gave similar results. **d**, Effect of 100 μg ml⁻¹ sCR1 on four parasite clones/lines. Rosetting is expressed as a percentage of the control rosette frequency for each clone/line without added sCR1. Data shown are mean and standard error from three (R29R⁺, PAR⁺ and MCR) or four (TM284) independent experiments.

in the process and may contribute to a more complex multi-molecular interaction involving PfEMP-1. Although rosetting is associated with severe disease in most studies, the present data cannot distinguish between the possibilities that rosetting itself contributes to pathology by exacerbating microvascular obstruction or that it represents a surrogate marker for some other adhesive interaction with, for example, endothelial cells. Further insight into the molecular mechanisms involved may lead to new treatments and prevention of severe *P. falciparum* infections. □

Methods

Parasite culture. Parasites were grown as described¹⁴. *P. falciparum* used were the clones R29 (ref. 14) and PAR⁺ (ref. 6), and the lines MCR¹⁸, TM180 (ref. 22), and TM284 (ref. 22).

Gelatin sedimentation. Sedimentation of parasite culture in plasmagel (3% gelatin in isotonic saline) results in separation of rosetting and non-rosetting infected RBC¹³. The upper non-rosetting fraction is completely free of rosetting infected RBC, whereas the bottom fraction is predominantly rosetting infected RBC but always contains a small proportion of non-rosetting infected RBC. The weak band from RNA of the rosetting infected RBC with R⁺ primers (Fig. 1c) reflects this contamination of the R⁺ fraction.

RT-PCR. RNA extraction and RT-PCR of *var* gene DBL-1 were done as described¹⁰: upstream primer (primer 1), 1'GC(T/C/A)TG(T/C)GCICIT(T/A)(C/T)(C/A)G, which is specific for DBL-1; downstream primer, UNIEBP3', 5'-CCA(A/T)C(T/G)(T/G)A(A/G)(A/G)AATTG(A/T)GG, which recognizes all DBL domains²⁷. The R29R⁺var1-specific primers were 5'-TGC ACC ATT CAG AAG ACA and 5' TCC TTG ACT TGT AAA AGC. The R29R⁺var3-specific primers were 5' CGA CGT CTA CAT CTA TGT and 5' ATT TTT CGC CTG TAG GTT. For the specific primers, 30 cycles of amplification were performed at 94 °C for 30 s, 55 °C for 30 s and 65 °C for 1 min 30 s.

COS-7 cell expression and RBC binding. Constructs for the expression of individual DBL domains as chimaeric proteins with the herpes simplex glycoprotein D signal sequence and transmembrane region were made using the pRE4 vector as described¹⁶. pR29R⁺dbl1 extends between nucleotides 244–1,377 (numbered from the first ATG of R29R⁺var1), pR29R⁺cidr from 1,276–1,328, pR29R⁺dbl2 from 2,326–3,207, pR29R⁺dbl3 from 3,391–4,389, and pR29R⁺dbl4 from 4,774–6,117. COS-7 cells were grown on 12-mm coverslips in 35-mm wells as described¹⁶ and transfected with 1 µg DNA per well using Lipofectamine (Life Technologies). Surface expression was detected by immunofluorescence assay using the monoclonal antibody DL6 to the pRE4 vector¹⁷. Binding assays were carried out with RBC at 50% haematocrit in binding medium (RPMI without bicarbonate, with 10% human serum) for 2 h at room temperature. Coverslips were washed gently with PBS0.1% BSA to remove non-adherent RBC and viewed microscopically. A COS-7 cell was scored as positive when adherent RBC covered more than 50% of the cell surface. The number of positive cells in 50 fields at 200× magnification was counted.

Rosetting of RBC variants. Infected RBC were purified by heparin disruption of rosettes, followed by centrifugation through a Percoll gradient¹³ and were stained with 20 µg ml⁻¹ ethidium bromide. The test RBC were thawed on the day of the experiment and labelled with the fluorescent dye PKH26 (ref. 28). Control experiments showed that freezing and thawing and labelling with PKH26 did not impair the ability of normal RBC to form rosettes. The test RBC were mixed with purified infected RBC and incubated at room temperature for 30 min on a plate shaker at 300 r.p.m. The rosette frequency (percentage of mature infected RBC in rosettes) was assessed by fluorescence microscopy. A minimum of 100 infected RBC were counted and scored for rosetting, a rosette being defined as the binding of two or more labelled RBC. The null/negative RBC tested were: Knops null (2 donors), Kell null (Ko), Bombay, Rh null, LW (a⁻b⁻), McLeod, Kidd null (Jk-3), Duffy (a⁻b⁻), Gerbich (-1, -2, -3), Lutheran (a⁻b⁻), Gregory (a⁻), JMH null, M^kM^k, Scianna (-1, -2), P (Tj^a-), Colton (a⁻b⁻), Adult i, Chido negative, Lewis (a⁻b⁻), Xg negative, Diego (b⁻), Lan negative, and Vel negative.

Rosette inhibition with sCR1. sCR1 was a gift from D. Fearon²³. Parasite cultures were stained with ethidium bromide and washed once and resuspended at 4% haematocrit in complete RPMI with 10% heat-inactivated human serum. An equal volume of sCR1 in RPMI was added and the rosettes disrupted by passing the suspension six times through a 25-gauge needle. R29

was incubated with doubling dilutions of sCR1, giving a range of final concentrations from 500 µg ml⁻¹ to 1.95 µg ml⁻¹. MCR⁺, PAR⁺ and TM284 were tested at 100 µg ml⁻¹ of sCR1. Cultures were incubated at room temperature for 30 min, then assessed by fluorescence microscopy, with 200 infected RBC counted and scored for rosetting.

Determination of CR1 copy number and SI(a) type. The mean CR1 copy number per RBC was determined by a double-antibody capture immunoassay using solubilized erythrocyte membranes²¹. SI(a) type was determined by the antiglobulin technique²⁹ using human antisera to SI(a).

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Correspondence and requests for materials to either J.A.R. (arowe@worf.molbio.ox.ac.uk) or L.H.M. (louis_miller@nih.gov). Sequences have been lodged with Genbank under accession numbers Y13402 (R29R⁺ var1 exon I); Y13403 (R29R⁺ var1 exon II); Y13404 (R29R⁺ var2); Y13405 (R29R⁺ var3); Y13406 (R29R⁺ var4); Y13407 (R29R⁺ var5); Y13408 (R29R⁺ var6).

Expression cloning of new receptors used by simian and human immunodeficiency viruses

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Several members of the chemokine-receptor family serve, in conjunction with CD4, as receptors for the entry of human immunodeficiency virus type I (HIV-1) into cells^{1–6}. The principal receptor for entry of macrophage-tropic (M-tropic) HIV-1 strains is CCR5, whereas that for T-cell-line-tropic (T-tropic) strains is CXCR4. Unlike HIV-1, infection with either M-tropic or T-tropic strains of simian immunodeficiency virus (SIV) can be mediated by CCR5, but not CXCR4 (refs 7–10). SIV strains will also infect CD4⁺ cells that lack CCR5, which suggests that these strains use as yet unidentified receptors^{7,9,10}. Here we use an expression-cloning strategy to identify SIV receptors and have isolated genes encoding two members of the seven-transmembrane G-protein-coupled receptor family that are used not only by SIVs, but also by strains of HIV-2 and M-tropic HIV-1. Both receptors are closely related to the chemokine-receptor family and are expressed in lymphoid tissues. One of the receptors is also expressed in colon and may therefore be important in viral transmission. Usage of these new receptors following experimental infection of non-human primates with SIV strains may provide important insight into viral transmission and the mechanisms of SIV- and HIV-induced acquired immune-deficiency syndrome.

To identify the receptors used by SIV, we used an HIV-luciferase vector pseudotyped with SIV envelope glycoproteins (Env) to infect several CD4⁺ cell lines². A murine 3T3.CD4 cell line expressing CCR5, but not CXCR4, was readily infected with viruses bearing Env proteins from either Rhesus macaque or African green monkey strains (SIV_{mac1A11} (ref. 11) or SIV_{agmTY01} (ref. 12), respectively) (Fig. 1a). The human astroglial U87.CD4 cell line, which does not express CCR5 or CXCR4 and is resistant to infection with pseudotypes displaying T-tropic HXB2 or M-tropic JRFL Env glycoproteins¹³, was readily infectable with SIV_{agmTY01} and, to a lesser degree, with SIV_{mac1A11} Env-pseudotyped viruses (Fig. 1b). In contrast, CEM × 174, a T-cell/B-cell hybrid that does not express CCR5 either and is commonly used to amplify SIV in culture¹⁴, was preferentially infected with SIV_{mac1A11}-pseudotyped virus (Table 1). The pattern was similar in the Hut78 T-cell line (Table 1), suggesting the presence of at least two additional receptors for SIV.

To isolate the genes encoding such receptors, we used an expression-cloning strategy (Fig. 2a). A human T-cell clone cDNA library, subcloned in the retroviral vector pMX (obtained from T. Kitamura, DNAX), was transfected into BOSC23 packaging cells and the supernatant from these cells was then used to infect 3T3.CD4 cells. The transduced 3T3.CD4 cells were in turn challenged with a selectable replication-defective virus (HIV-puro) pseudotyped with either the SIV_{agmTY01} or SIV_{mac1A11} Env glycoproteins, followed by puromycin selection. A total of 280 puromycin-resistant colonies were obtained with SIV_{agmTY01} infection and about 170 with SIV_{mac1A11} infection. These colonies were then tested for their ability to be infected with HIV-luc pseudotyped with the HIV-1 M-tropic Env protein JRFL. Representative results after infection of several

candidate colonies with virus bearing SIV or HIV Env glycoproteins are shown in Fig. 2b. About 10% of the SIV_{agmTY01}-selected and 90% of the SIV_{mac1A11}-selected colonies were infectable with the JRFL Env pseudotypes, and all were shown by polymerase chain reaction (PCR) and antibody staining to express CCR5 (for example, clones C1.193 and C2.219 in Fig. 2b). Genomic DNAs extracted from CCR5-negative colonies were subjected to PCR amplification using the primers flanking inserts in the retroviral vectors. A common 2.1-kilobase (kb) band was detected with SIV_{agmTY01}-selected colonies, and a common 2.2-kb band with SIV_{mac1A11}-selected colonies. The PCR products were subcloned into expression vector pCR3.1 (Invitrogen) and the DNA sequenced. The complementary DNA selected with the SIV_{agmTY01} pseudotypes was found to encode a new protein of 342 amino acids, which we named Bonzo, whereas its counterpart selected with the SIV_{mac1A11} pseudotypes encodes a protein of 360 amino acids, designated BOB (for brother of Bonzo). Comparison of these sequences with those in genome databases indicates that both molecules are members of the large family of G-protein-coupled receptors. BOB is identical to a previously cloned orphan receptor, GPR15 (ref. 15), whereas Bonzo has no identity to

Table 1 Infection of T-cell lines with SIV isolates

Cell line	Luciferase activity (c.p.s. × 10 ⁻³)		
	No Env	mac1A11	agmTY01
CEM	<1	21	736
CEM × 174	<1	2,025	191
Hut78	<1	145	51

Infection of transformed CD4⁺ cell lines was done by using HIV-luciferase-reporter virus pseudotyped with SIV Env proteins. Values are the average of duplicate determinations. Standard errors were less than 15% of the average value.

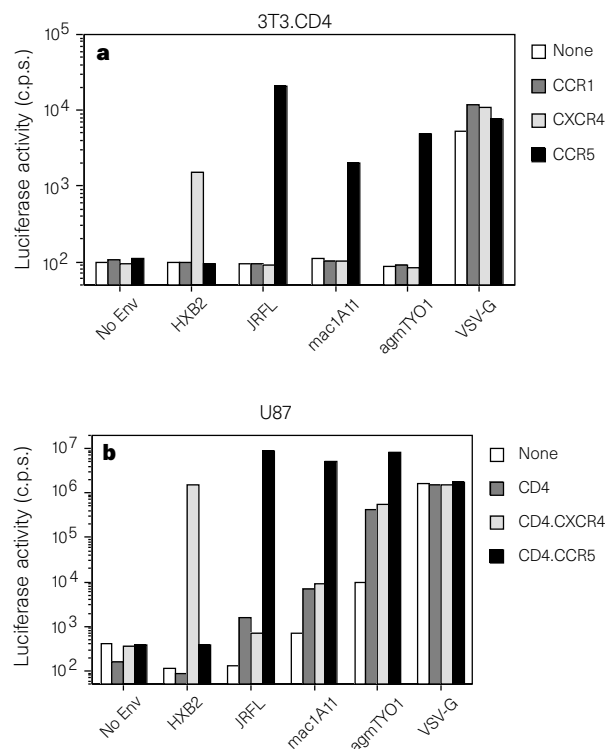


Figure 1 Specificity of SIV Envs for chemokine receptors on target cells. **a**, Infection of 3T3.CD4 cells stably expressing CCR1, CCR5, or CXCR4 with luciferase reporter viruses pseudotyped with Env proteins of HIV-1 (HXB2 and JRFL), SIV (mac1A11 and agmTY01) or VSV-G. **b**, Infection of U87 cells and U87.CD4 cells with the pseudotyped reporter viruses. Lysates were assayed 3 days post-infection for luciferase activity as described in Methods.

sequences in the expressed-sequence tag (EST) databases. Both BOB and Bonzo are related to chemokine-receptor family proteins but share only 25–30% amino-acid sequence identity with CCR5 and CXCR4. Sequence alignments of these receptors and of CCR5 and CXCR4 are shown in Fig. 2c. Using specific primers, we also cloned homologues of the SIV receptors from African green monkey and pigtail macaque. These were found to be 94–97% identical to the human sequences (data not shown; sequences are lodged with Genbank).

To test the viral-receptor function of the newly identified molecules, their cDNAs were cloned into the pBABE-puro expression vector and were transiently transfected into human 293T cells together with human CD4. Cells were separately transfected with vectors encoding CCR5 or CCR1 as controls. Transfected cells were then infected with HIV-luc viruses pseudotyped with Env glycoproteins from a diverse set of SIV, HIV-2 and primary HIV-1

isolates. As shown in Fig. 3a, the three SIV envelopes tested were all able to use Bonzo, BOB and CCR5. The inability of SIV Env-pseudotyped virus to infect CCR1-transfected control cells also shows that none of the SIV Env glycoproteins tested can use CXCR4, which is expressed endogenously in 293T cells. Most of the HIV-2 Env proteins tested were also able to use BOB and/or Bonzo (Fig. 3a). Several HIV-2 isolates that grow on transformed T-cell lines can also use CXCR4, explaining their high background in 293T cells (for example, ROD and UC2). Consistent with our previous results¹⁰, most of the HIV-2 Env proteins tested were able to use CCR5 to varying degrees, with the exception of UC2, which uses CXCR4 exclusively. The specificities of the different HIV-2 Env proteins have been confirmed in infections of 3T3.CD4 cells, as discussed later (Fig. 3c, and data not shown).

We next tested a panel of HIV-1 Env glycoproteins whose tropism for CCRs and CXCR4 had been determined. Several CCR5-tropic

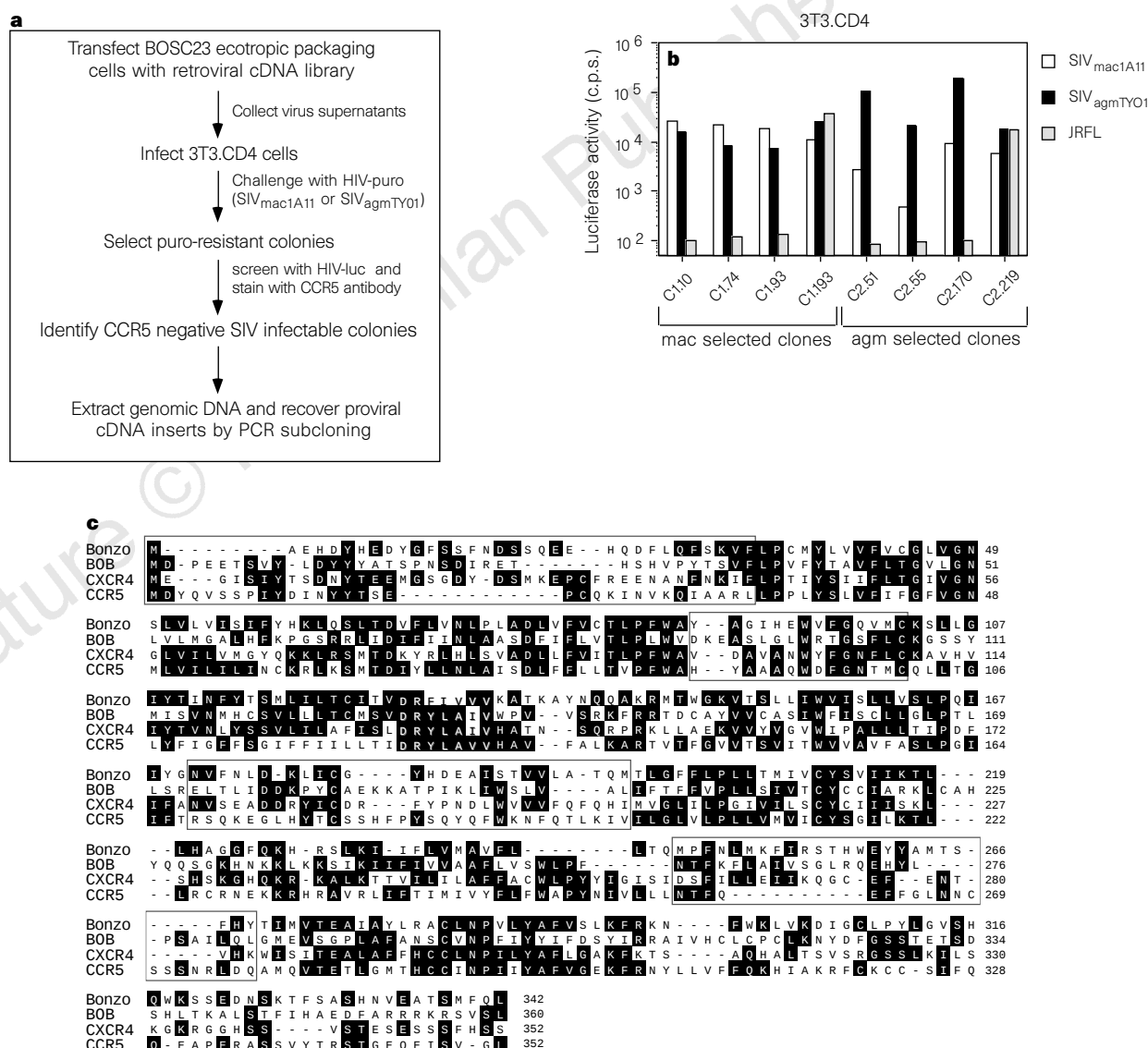


Figure 2 Expression cloning of novel SIV receptors. **a**, Outline of the expression cloning strategy used to isolate SIV receptors. **b**, Infection of representative 3T3.CD4 clones, previously selected with HIV-puro(SIV) pseudotyped virus, with HIV-luc pseudotyped with SIV and M-tropic HIV-1 Env proteins. **c**, Amino-acid sequence alignment of the receptors encoded by the BOB and Bonzo cDNAs with HIV receptors CXCR4 and CCR5. Identical amino acids are shaded and the

putative extracellular domains (based on CCR5 alignment) are boxed. The 'DRY box' sequence, which is involved in signalling and is characteristic of chemokine receptors, is highlighted in bold. Bonzo contains a divergent 'DRY box' motif compared to other HIV receptors; this sequence has thus been observed in only one other chemokine receptor, EBI 2.

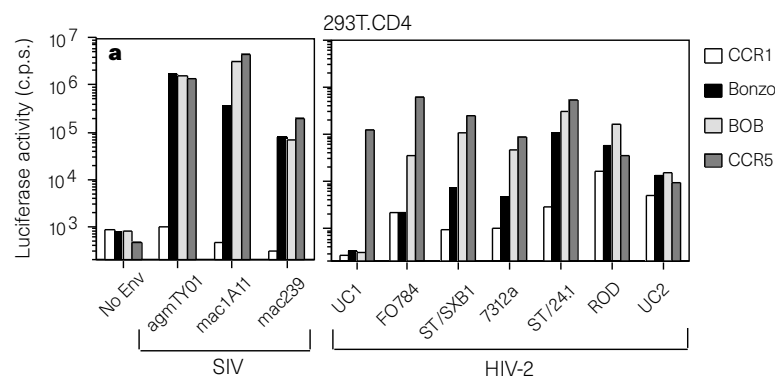
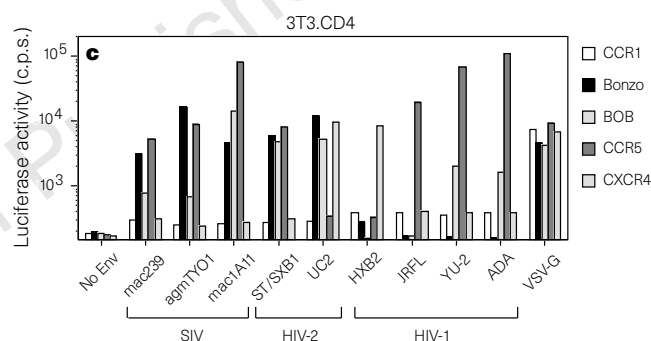
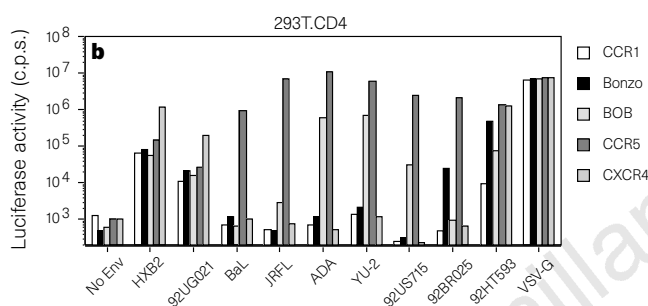


Figure 3 BOB and Bonzo mediate entry of SIV, HIV-2 and M-tropic or dual-tropic HIV-1 strains. **a**, Infection of 293T cells transiently cotransfected with plasmids encoding CD4 plus CCR1, Bonzo, BOB or CCR5. The cells were infected 2 days later with luciferase-reporter viruses pseudotyped with different SIV or HIV-2 Env proteins. The HIV-2 Env proteins, UC1, FO784, ST/SXB1 and 7312a have been shown to use CCR5; ST/24.1 and ROD can use both CCR5 and CXCR4; UC2 uses only CXCR4. **b**, Infection of the transiently transfected 293T cells with HIV-luc pseudotyped with HIV-1 Env proteins or control VSV-G. Target cells expressing CXCR4 were also included to identify T-tropic Env proteins, even though 293T cells express endogenous CXCR4. **c**, 3T3.CD4 cells stably expressing CCR1, BOB, Bonzo, CCR5 or CXCR4 were infected with the luciferase reporter viruses as described. Luciferase activity was measured on day 3. Similar results were obtained in three independent experiments.



Env glycoproteins (ADA and YU-2, which can also use CCR3 (refs 6, 16), 92US715 and 91US005) and one dual-tropic for CXCR4 and R5 (92HT593) were also able to infect cells transfected with BOB (Fig. 3b, and data not shown). Two of the Env proteins from primary HIV-1 strains (the dual tropic 92HT593 and the CCR5-tropic 92BR025) also utilized Bonzo (Fig. 3b). None of the three T-tropic envelopes tested (HXB2, 92UG021 or 92HT599) was found to use BOB or Bonzo. Several other CCR5-tropic Env proteins (BaL, 92UG975, 93HT966, 92RW020, 93TH976, 92BR020 and 93MW965) did not use these receptors either, and one (JRFL) used BOB only weakly (Fig. 3b, and data not shown).

Infection of 293T.CD4 cells with viruses using CXCR4 results in a high background owing to expression of endogenous CXCR4 (for example, HIV-2 UC2 in Fig. 3a). We therefore stably introduced Bonzo, BOB, CCR5 and CCR1 into mouse 3T3 cells expressing human CD4, and infected these with the panel of HIV-luc pseudotype viruses (Fig. 3c). The infection results were consistent with those obtained from the transient transfections of 293T cells. The SIV_{mac1A11}-pseudotyped virus infected cells expressing BOB more effectively than cells expressing Bonzo, whereas the converse was observed with virus bearing SIV_{agmTYO1} Env. Infection of human osteosarcoma cells, expressing CD4 and either BOB, Bonzo or CCR5, with replication-competent SIV_{agm} and SIV_{mac} strains yielded similar results (data not shown).

We next determined the expression pattern of the newly identified receptors in normal human tissues using northern blot hybridization (Fig. 4a). Bonzo cDNA hybridized to a 2.1-kb mRNA species expressed at high levels in spleen, thymus, small intestine and, to a lesser extent, in peripheral blood leukocytes (PBL), prostate and colon. In the placenta, a 2.6-kb species was detected; whether this represents a different homologous gene product, the product of differential splicing, or an antisense messenger RNA is unknown. Similarly, BOB mRNA was detected in spleen and PBL; expression was weaker in thymus and small intestine. BOB mRNA

was present in colon, where its high expression indicates that it may be expressed in non-lymphoid cells. It will therefore be important to determine whether this expression pattern is relevant for sexual transmission of HIV.

Expression of the newly identified receptors was also analysed in peripheral blood mononuclear cells (PBMC) and in FACS-purified subsets by PCR with reverse transcription (RT-PCR) (Fig. 4b). Expression of Bonzo mRNA was evident in unstimulated and phytohaemagglutinin (PHA)-stimulated PBMC, T cells and monocytes, but not in B cells. BOB mRNA was detected in PHA-stimulated PBMC, purified T cells, and weakly in unstimulated PBMC. However, expression was either minimal or absent in monocytes and B cells.

We next investigated whether susceptibility of various cell lines to infection with SIV could be explained by the expression of the new receptors. Northern blot analysis of RNA from cell lines shows that CEM × 174 cells, the parental 174 cells, and Hut78 cells express high levels of BOB but not of Bonzo (Fig. 4c). This pattern of expression correlates with the preferential infection of CEM × 174 and Hut78 cells by the SIVs from rhesus macaque. Low levels of Bonzo mRNA could be detected in CEM and U87 cells, both by northern blotting (Fig. 4c) and by RT-PCR (data not shown). Furthermore, antiserum raised against the N-terminal sequence of Bonzo blocked infection of U87.CD4 and CEM cells with HIV-luc pseudotyped with SIV Env glycoproteins (data not shown). The expression pattern of Bonzo thus probably explains the ability of these cells to be infected with African green monkey strains of SIV.

In humans, CCR5-tropic viruses are primarily involved in transmission, whereas viruses with broader tropism, particularly for CXCR4, emerge during the progression to immunodeficiency¹⁷. It is not yet known whether appearance of CXCR4-tropic viruses is a consequence or cause of the decline of the immune system. Insight into this key problem of virus evolution is likely to require experimentation in animal models. Infection of non-human pri-

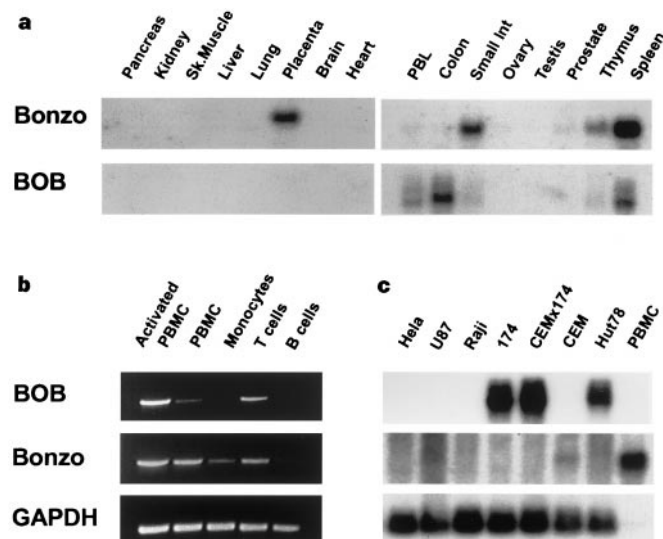


Figure 4 Expression of BOB and Bonzo mRNAs in human lymphoid tissues and cell lines. **a**, RNA tissue blot of various human tissues (Clontech) probed with full-length BOB and Bonzo cDNAs as described in Methods. The signals correspond to 2.1-kb Bonzo and 2.2-kb BOB transcripts. **b**, RT-PCR analysis of BOB and Bonzo transcripts in human PBMC, resting or stimulated with PHA (Sigma) for 7 days, and purified lymphocyte subsets. Total RNAs isolated from these cells were amplified with primers specific for BOB, Bonzo or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control (bottom). Primer specificity has been verified on 3T3 cells expressing BOB or Bonzo. **c**, Northern blot analysis of poly(A)⁺ mRNA from different cell types. Each lane was loaded with 5 µg poly(A)⁺ mRNA, except for the PBMC lane, which contained 10 µg total RNA.

mates with SIV is currently the only good animal model for studying the pathogenesis of the immunodeficiency viruses¹⁸. Moreover, different species of non-human primates vary widely in their responses to SIV infection. For example, Rhesus macaques succumb to immunodeficiency that closely resembles AIDS in humans, but sooty mangabeys and African green monkeys can sustain infection with little evidence of immune-system damage¹⁹. These interspecies differences may provide important clues for understanding and containing disease progression in HIV-infected humans. Although SIV viruses do not seem to use CXCR4, they use CCR5, Bonzo and BOB as receptors. Variations in the structure or expression of these molecules may represent host variables that influence the outcome of SIV infection. It will therefore be important to determine whether the ability of primary strains of SIV to use the newly described receptors correlates with the susceptibility of animals to progressive disease.

The finding that envelope glycoproteins from both HIV-1 and HIV-2 primary strains can use Bonzo and BOB as receptors raises the possibility that these receptors may be important in infection of humans. Recent reports have also described CCR5^{-/-} homozygous individuals who are infected with HIV-1 (refs 20–22). As several M-tropic strains can efficiently use the new receptors, especially BOB, these receptors may substitute for CCR5 in establishing HIV-1 infection. However, these individuals may be infected with CXCR4-tropic viruses. SIV can replicate in human CCR5-deficient PBMC⁷, which on the basis of our results can be explained by the ability of SIVs to use Bonzo and/or BOB as receptors in the mutant cells; it will be important to determine whether HIV-1 recovered from infected CCR5-negative individuals can also use these newly identified receptors.

There are now six members of the chemokine-receptor family that function in HIV-1 entry: CCR5, CXCR4, CCR2b, CCR3, Bonzo and BOB. Application of the expression-cloning approach

described here should identify additional receptors if primary HIV-1 Env glycoproteins and expression libraries from other tissues are used in the screening. The ability of HIVs to infect cells by way of the newly described receptors, which are expressed in human T lymphocytes and monocytes, may complicate strategies aimed at blocking CCR5-tropic virus entry. To understand the role of these receptors in infection and in the progression of disease will require the ligands for Bonzo and BOB to be identified and the biological functions of the receptors to be elucidated.

Note added in proof: Since this manuscript was submitted, S. Liao *et al.*³¹ have reported on HIV-1 receptor, STRL33, which is the same as Bonzo. □

Methods

Expression cloning. A human T-cell clone cDNA library L10/MX, in the retroviral vector pMX, was provided by T. Kitamura (DNAX). High-titre retroviruses, produced by transient transfection of the library into the packaging cell line BOSC23 (ref. 23), were used to infect NIH3T3 cells stably expressing human CD4. Briefly, 10 µg retroviral plasmid DNA was transfected into BOSC23 cells (2×10^6 cells in a 10-cm culture dish) by calcium phosphate precipitation. Two days post-transfection, the culture supernatant was cleared of cell debris and added with 8 µg ml⁻¹ polybrene to ten 10-cm dishes, each containing 5×10^5 3T3.CD4 cells. After 16 h incubation at 37 °C, the viral supernatant was replaced with DMEM containing 10% fetal calf serum. This high multiplicity of infection resulted in 100% transduction of the 3T3.CD4 cells, as assayed in controls in which pMX.mCD4, whose product is detectable by FACS analysis, was mixed in various ratios with the cDNA library. The library-transduced 3T3.CD4 cells were then transferred to 15-cm plates and infected with a selectable replication-defective virus, HIV-puro, pseudotyped with either SIV_{agmTY01} or SIV_{mac1A11} Env proteins. HIV-puro pseudotypes were generated by transfecting 293T cells with 10 µg pHIV-puro (an HIV construct in which *env* contains a frameshift mutation and *nef* has been replaced by the SV40 promoter regulating the puromycin-resistance gene, kindly provided by R. Sutton) and 10 µg Env-expressing plasmids. Virus-containing supernatants were collected 48 h post-transfection, filtered, and applied to the library-transduced 3T3.CD4 cells (10 ml viral supernatant per plate). After two days, the 3T3.CD4 cells were replated in 24-well plates (1×10^5 cells per well) with medium containing 5 µg ml⁻¹ puromycin. Selection medium was changed every 2 to 3 days, and visible colonies arose typically in 6 to 9 days. These colonies were then tested for their ability to be infected with HIV-luc pseudotyped with the HIV-1 M-tropic Env JRFL or SIV Env proteins from mac1A11 or agmTY01. Colonies infected by JRFL pseudotyped viruses were also stained with anti-CCR5 antibody (gift from C. Mackay).

Genomic DNA isolation and PCR cloning. To recover retrovirus-transduced cDNAs, 50 ng genomic DNA isolated from each puromycin-resistant clone was subjected to PCR. cDNA segments were amplified by using primers complementary to retroviral vector flanking the inserts (upstream, 5'-GGTGGACCATTCTCTAGACT; downstream, 5'-CCCTTTTCTGAGAC-TAAAT). The PCR was set up using the Expand kit from Boehringer-Mannheim and run for 35 cycles at 58 °C annealing temperature. The resulting PCR fragment was purified and cloned into the pCR3.1 vector (Invitrogen) and sequenced. Amino-acid sequence alignments were analysed using the DNASTAR program.

Pseudotyped virus infection assays. Luciferase reporter viruses were prepared in 293T cells as described^{2,24} using pNL-Luc-ER⁺ vector and Env expression plasmids. Env expression vectors for HIV-1 JRFL, ADA, BaL, HXB2, 92UG021, 92US715, 92BR025, 92HT593, as well as SIV mac239, mac1A11 and agmTY01 have been described^{11,12,24–27}. The HIV-2 ST24.1 and p7312A envelope expression plasmids were generated by PCR subcloning of the proviral DNA template (gifts from B. Hahn) into the expression plasmid pSP272. The HIV-2 UC1 and UC2 (ref. 28) envelope expression plasmids were from C. Weiss. Proviral DNAs from YU-2, HIV-2 ST/SXB1 (ref. 29) and FO784 (ref. 30) were used in cotransfection with pNL-Luc-ER⁺ vector to generate mixed viral particles containing both wild-type and luciferase-containing particles. Pseudotyped viruses in supernatants of transfected 293T cells were quantified by p24 ELISA (Coulter) to normalize virus stocks for infection. 293T cells transiently cotransfected with pMX-CD4 and with pBABE-puro expressing

CCR1, BOB, Bonzo, CCR5 or CXCR4 (ref. 2) were infected with pseudotyped viruses (50 ng p24 per infection, 5×10^4 cells per well in 24-well plates). 3T3.CD4 cells that stably express the different chemokine receptors² were similarly infected. 3T3.CD4 cells expressing the new receptors were prepared by retroviral transduction with pMX.Bonzo and pMX.BOB. After 3 days, cells were resuspended in 120 μ l of luciferase lysis buffer (Promega). The luciferase activity in 20 μ l lysate was assayed in a Wallac Microbeta 1450 Counter using commercially available reagents (Promega).

Northern blots. Polyadenylated RNA was prepared from various human cell lines with the Micro-Fast Track kit (Invitrogen). Samples (5 μ g RNA) were electrophoresed through a 1% agarose-formaldehyde gel and transferred to a GeneScreen nitrocellulose membrane. Multiple-tissue northern blots I and II, purchased from Clontech, contain $\sim 2 \mu$ g poly(A)⁺ RNA from each tissue. Integrity of blots was assayed by GAPDH probing. Full-length cDNAs of BOB and Bonzo were labelled with ³²P by using a Random Primed DNA-labelling kit (Boehringer-Mannheim) and used to probe northern blots.

Lymphocyte purification and RT-PCR. Monocytes were purified from buffy coats using a 46% Percoll gradient. To purify T- and B-cell subsets, PBMC were stained with phycoerythrin-conjugated anti-CD3 or anti-CD19 antibodies (Becton-Dickinson) and sorted using FACS (Coulter). Total RNA was isolated using RNazol reagent (Cinna/Biotech), treated with RNase-free DNase, and 0.5 μ g was taken for cDNA synthesis using Superscript II RNase H reverse transcriptase and random hexamer primers (Gibco-BRL); one-twentieth of this reaction was used as a template for PCR amplification with *Taq* DNA polymerase. BOB primers used for RT-PCR: upstream (from ATG) 5'-CATCTGCTCTTTGGTGATG; downstream (550 bp from ATG), 5'-GTATGGCTTATCATCAATCAGC, amplifies ~ 600 bp of transcript; Bonzo primers were: upstream (from 270 bp downstream of ATG), 5'-CAGGCATC-CATGAATGGGTGT, and downstream (from the stop codon), 5'-CAAGGCC-TATAACTGGAACATGCTG, amplifies ~ 750 bp of transcript. The PCR reaction was run for 30 cycles at 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min. To exclude contamination of genomic DNA, control cDNA reactions in which reverse transcriptase was omitted were prepared in parallel. These were uniformly negative.

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X-linked IAP is a direct inhibitor of cell-death proteases

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The inhibitor-of-apoptosis (IAP) family of genes has an evolutionarily conserved role in regulating programmed cell death in animals ranging from insects to humans^{1–6}. Ectopic expression of human IAP proteins can suppress cell death induced by a variety of stimuli, but the mechanism of this inhibition was previously unknown. Here we show that human X-chromosome-linked IAP directly inhibits at least two members of the caspase family of cell-death proteases, caspase-3 and caspase-7. As the caspases are highly conserved throughout the animal kingdom and are the principal effectors of apoptosis⁷, our findings suggest how IAPs might inhibit cell death, providing evidence for a mechanism of action for these mammalian cell-death suppressors.

For our initial investigation of human X-chromosome-linked IAP (XIAP; hILP; MIHA), we used recombinant XIAP protein and a cell-free system in which cytochrome *c* was added to cytosolic extracts. When added to normal cytosol, cytochrome *c* initiates an apoptotic programme which includes proteolytic processing and activation of certain caspases, and apoptotic-like destruction of exogenously added nuclei⁸. Release of cytochrome *c* from mitochondria into the cytosol appears to be commonly associated with apoptosis^{8,9}.

Purified nuclei mostly remained intact in the cytosol from controls. In contrast, adding cytochrome *c* caused apoptotic-like destruction of nearly all nuclei (Fig. 1A). Addition of cytochrome *c* directly to nuclei without cytosol had no effect, demonstrating the requirement for cytosolic factors (results not shown)⁸. Recombinant XIAP (rXIAP) added simultaneously with cytochrome *c* severely inhibited nuclear destruction (Fig. 1A). An equivalent

amount of added Bcl-2 protein had no protective effect.

In addition to nuclear destruction, XIAP also inhibited cytochrome-*c*-induced caspase activation. Two important proteolytic activities associated with caspases can be measured by assaying the cleavage of peptide substrates containing the sequences DEVD and YVAD: the former are recognized by most caspases and the latter by the ICE (caspase-1) subfamily (reviewed in ref. 10). Addition of cytochrome *c* to extracts prepared from human 293 epithelial cells caused a rapid accumulation of DEVD-specific protease activity (Fig. 1B) but not of YVAD-specific cleavage (results not shown). The rXIAP protein markedly reduced cytochrome-*c*-induced generation of DEVD-cleaving activity in these extracts, whereas many control proteins had little or no effect even at ≥ 10 -fold molar excess over rXIAP (Fig. 1B). Results were similar when DEVD-specific hydrolysis was measured for cytosol from Jurkat T-cells, indicating that these findings are probably not unique to a single cell line (Fig. 1B). Furthermore, in cytosolic extracts prepared from 293T cells two days after transient transfection with either pcDNA3-XIAP or pcDNA3 control plasmid, cytochrome-*c*-induced activation of DEVD-cleaving proteases *in vitro* was reduced by > 50% in the XIAP-overexpressing extracts compared to control extracts (not shown). Thus, both exogenously added rXIAP and endogenously produced XIAP inhibit cytochrome-*c*-induced caspase activation *in vitro*.

Addition of cytochrome *c* to cytosolic extracts causes proteolytic processing of caspase-3 (CPP32; Yama; apopain) from its ~32K zymogen precursor into active large and small subunits—a result we confirmed by immunoblotting using a rabbit antiserum specific for the zymogen and large subunit of this protease (Fig. 1C). In contrast, addition of rXIAP protein markedly inhibited cytochrome-*c*-induced cleavage of the caspase-3 zymogen (Fig. 1C). Equivalent concentrations of several control proteins (glutathione-S-transferase (GST), GST-Bcl-2, GST-Bax, GST-CD40) had no effect on caspase-3 processing (result not shown).

As an alternative way to induce apoptosis in these extracts, we added recombinant, active caspase-8 (FLICE; Mach; Mch5), a protease that associates with Fas and TNF-R1 receptor complexes and functions as an upstream initiator of proteolytic cascades that lead to caspase-3 activation and apoptosis^{11–14}. Caspase-8 stimulated cleavage of caspase-3, yielding large-subunit bands characteristic of protease activation (Fig. 1C). In the presence of XIAP, caspase-8 induced a partially processed form of caspase-3, presumably by cleavage between the large and small subunits without the subsequent removal of the N-terminal peptide 'prodomain'¹⁵. XIAP thus did not prevent the initial cleavage of caspase-3 induced (perhaps directly) by caspase-8, but did inhibit the subsequent processing to the mature large subunit. XIAP also inhibited caspase-8-induced destruction of nuclei and the accumulation of DEVD-specific protease in extracts (not shown), indicating that it interferes with a protease(s) downstream of caspase-8.

As XIAP prevents the completion of caspase-3 processing in extracts treated with caspase-8, XIAP might directly inhibit this protease, given that removal of the N-terminal prodomain of caspase-3 occurs by autocatalysis^{15,16}. We therefore tested the effect of rXIAP on the activity of several purified and active recombinant caspases *in vitro*. Purified GST-XIAP inhibited >95% of DEVD-peptide cleavage by caspase-3 and by the closely related caspase-7 (LAP3/Mch3) when added at a ~10-fold molar excess, but did not affect substrate cleavage by caspases 8, 6 (Mch2) or 1, even at 50-fold molar excess (Fig. 2a). Even a 2-fold molar excess of XIAP was sufficient to inhibit >70% of caspase-3 and ~100% of caspase-7 activity when assayed at high enzyme concentrations (0.5–0.7 μ M) and with XIAP added before 100 μ M DEVD-pNA substrate. A GST-fusion protein containing only the three BIR (baculovirus IAP repeat) domains of XIAP (residues 1–336) also potently inhibited caspase-3 and caspase-7 *in vitro*, whereas a GST fusion containing the RING domain (residues 337–497), as well as several control

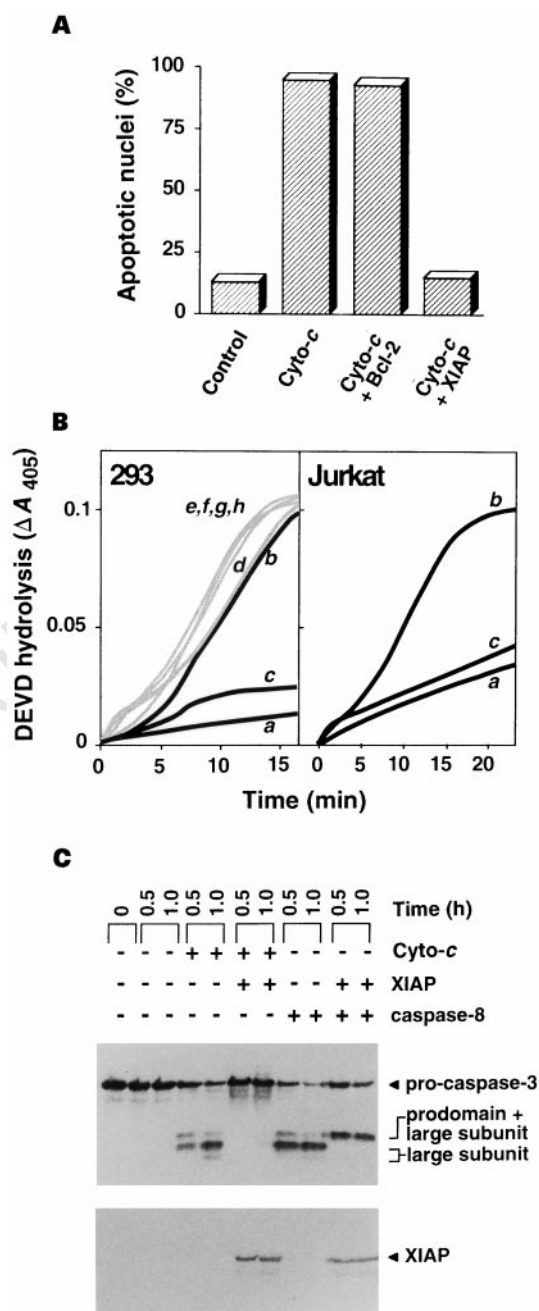


Figure 1 XIAP inhibits cytochrome-*c*-induced processing of caspase-3 and destruction of nuclei in cytosolic extracts. **A**, Isolated nuclei were added to cytosolic extracts, followed by 10 μ M cytochrome *c* and 1 mM dATP, with or without GST-XIAP (0.4 μ M) or GST-Bcl-2 (0.4 μ M), and the percentage of nuclei with apoptotic features was determined (data representative of 2 of 2 experiments). **B**, Cytosolic extracts from 293 or Jurkat cells were used as a control (**a**) or were treated with 1 μ M cytochrome *c* and 1 mM dATP alone (**b**), or with ~0.2 μ M GST-XIAP (**c**). Controls (grey lines) consisted of addition of ~10x more (~2 μ M) control GST-fusion proteins, including GST-Bcl-2 (**d**), GST-Bax (**e**), GST-NM23 (**f**) and GST-CD40 cytosolic domain (**g**), or addition of 5 μ M His₆-S5a protein (**h**). DEVD-pNA hydrolysis was then measured at various times (data representative of several experiments performed with independent GST-XIAP preparations). **C**, Immunoblot analysis for caspase-3 was performed using cytosolic extracts (50 μ g per lane) before or at 0.5 and 1.0 h after adding either cytochrome *c* and dATP or active caspase-8 proteases (~1 μ g), with (+) or without (–) ~0.2 μ M GST-XIAP (30- μ l reaction volumes). The positions are noted for the pro-enzyme, partially processed caspase-3 (large subunit + prodomain) and the large subunit forms of fully processed caspase-3. The blot was also probed with anti-XIAP antiserum (lower panel).

GST-fusion proteins, had no significant effect (not shown). The inhibition of caspase-3 and caspase-7 by XIAP was concentration-dependent (Fig. 2b,c). Based upon progress curve analysis, XIAP demonstrated tight, reversible binding to caspase-3 and caspase-7 with inhibition constants of ~ 0.7 and ~ 0.2 nM, respectively. These values compare favourably with viral inhibitors of caspases (cowpox CrmA: 0.01–0.95 nM; baculovirus p35: 1.0 nM for their target caspases)^{17,18}.

Besides inhibiting the proteolytic activity of caspase-3 and caspase-7, XIAP also bound directly to these proteases *in vitro*. For example, mixing purified GST–XIAP protein or GST–CD40 (negative control) immobilized on glutathione–Sephadex with purified recombinant caspases revealed specific binding of XIAP to caspase-3 and caspase-7 but not to caspases 1, 6 or 8 (Fig. 2d, and data not shown). Specific binding to caspase-3 and caspase-7 was also observed when *in vitro* translated ³⁵S-labelled Myc-tagged XIAP was tested for binding to His₆-tagged active proteases immobilized

on Ni resin (not shown). XIAP, however, did not bind efficiently to the unprocessed pro-enzymes, based on similar experiments in which immobilized GST–XIAP was assayed for binding to unprocessed caspase-3 and caspase-7 in cytosolic extracts (Fig. 2e,f). In contrast, after treatment of the same extracts with cytochrome c, processed caspase-3 and caspase-7 then bound to immobilized GST–XIAP but not GST–CD40. The partially processed caspase-3 seen in extracts treated with the combination of caspase-8 and rXIAP (Fig. 1C) also bound efficiently to immobilized GST–XIAP (not shown), consistent with the idea that cleavage between the large and small subunit but not removal of the prodomain is required for protease activation. Taken together, these data indicate that XIAP directly binds active caspase-3 and caspase-7, but not the inactive zymogens.

To determine whether XIAP can inhibit processing and activation of caspases in intact cells, we did transient transfection experiments in human 293T cells using overexpression of Bax protein as a

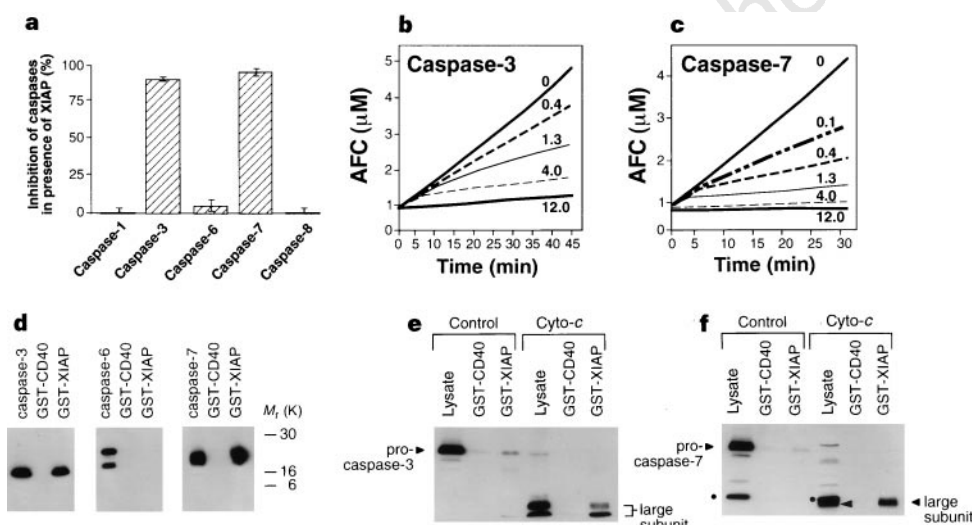


Figure 2 XIAP directly binds and inhibits caspase-3 and caspase-7. **a**, Purified recombinant caspases 1, 3, 6, 7 and 8 (1–10 nM) were incubated with or without up to a 50-fold molar excess of XIAP (10–50 nM range). Data are expressed as per cent inhibition, based on the average ratio of velocities (v_i/v_0) of three independent experiments (mean $\pm$ s.e.) performed in the presence (v_i) or absence (v_0) of GST–XIAP. **b, c**, Representative progress curves are shown for caspase-3 (0.05 nM) and caspase-7 (0.1 nM), respectively, in which XIAP was added at various concentrations (nM) simultaneously with substrate (100 μM DEVD-AFC). **d**, GST–XIAP or

GST–CD40 on glutathione–Sephadex were tested for binding to active recombinant proteases by immunoblotting. As a control, purified proteases (0.1 μg) were run directly in the gel. **e, f**, GST–XIAP or GST–CD40 fusion proteins immobilized on glutathione–Sephadex were assayed by immunoblotting for binding to caspase-3 (**e**) or caspase-7 (**f**) in untreated ('control') 293 cytosolic extracts or after activation with cytochrome c. As a control, lysates (50 μg) were run directly in gels. A nonspecific band detected by the caspase-7 antiserum in cell lysates is indicated by a dot to distinguish it from the processed ~ 20 K large subunit.

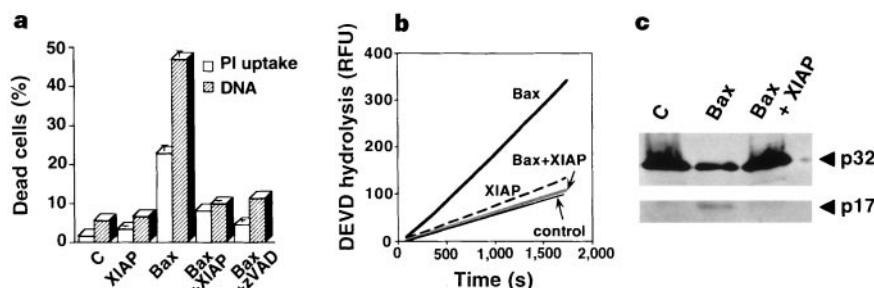


Figure 3 XIAP inhibits Bax-induced caspase-3 processing and cell death in 293T cells. **a**, The percentage of dead (uptake of propidium iodide) and apoptotic (*hypodiploid DNA content) cells (mean $\pm$ s.e. for 3 determinations) was determined 1 day after transfection of 293T cells with pcDNA3 ('C'), pcDNA3-Bax, pcDNA3-Myc-XIAP, or pcDNA3-Bax and pcDNA3-Myc-XIAP. ZVAD-fmk (50 μM)

(Bachem) was added immediately after Bax-transfection. Extracts were prepared from transfected cells for either **b**, protease assay (DEVD-AFC cleavage activity; expressed as relative fluorescence units, RFU), or **c**, immunoblot analysis of caspase-3 processing. Different exposure was used for 32K pro- and 17K processed caspase-3.

stimulus for inducing apoptosis and caspase-3 activation. Previously Bax had been shown to induce mitochondrial permeability transition (predicted to cause the release of cytochrome *c*) and processing of caspases 3, 6 and 7 (ref. 19). Transfection of a Bax-encoding plasmid into 293T cells caused an approximately 8–10-fold increase in cell death (as measured by trypan-blue or propidium iodide uptake) about an 8–10-fold increase in apoptosis (as measured by DNA fragmentation) compared to control transfected cells (Fig. 3a). In contrast, when Bax- and XIAP-encoding plasmids were co-transfected, Bax-mediated cell death and apoptosis were inhibited ($P < 0.01$; *t*-test). Immunoblotting confirmed that the Myc-tagged XIAP protein was expressed in the expected transfected cells and verified that XIAP did not inhibit production of Bax protein (not shown), implying that XIAP blocks an event downstream of Bax. A Myc-tagged version of XIAP containing only the BIR domains was as effective as the full-length protein at suppressing Bax-induced apoptosis, whereas the RING domain of XIAP was inactive (the percentages of apoptotic cells, based on hypodiploid DNA content, were $11 \pm 1\%$ (neo control), $53 \pm 2\%$ (Bax), $15 \pm 0.5\%$ (Bax + XIAP), $18 \pm 1\%$ (Bax + BIRs), $47 \pm 1\%$ (Bax + RING)). The caspase-inhibiting peptide zVAD-fmk also inhibited Bax-induced apoptosis in 293T cells, consistent with a role for caspases in Bax-triggered cell death in these cells.

Extracts prepared from Bax-transfected 293T cells also contained much more caspase activity and processed caspase-3 than did control transfected cells. In contrast, analysis of extracts from cells co-transfected with Bax and XIAP revealed that XIAP markedly inhibited Bax-induced generation of caspase activity (Fig. 3b) and caspase-3 processing (Fig. 3c). This suppression of caspase-3 processing in cells (Fig. 3c) and cytosolic extracts *in vitro* (Fig. 1c) implies that XIAP either blocks the activity of caspases upstream of caspase-3 or that it prevents caspase-3-mediated processing of procaspase-3.

We have described, to our knowledge for the first time, a mechanism of action for an IAP-family protein: namely, inhibition of active cell-death proteases. Although viral proteins such as CrmA and p35 bind to and inhibit caspases, XIAP is the first cellular protein identified with these characteristics. The extensive homology shared by IAP-family proteins across evolution (particularly in the BIR domains) suggests that other members of this family of apoptosis-suppressing proteins also inhibit specific caspases. Although apoptosis seems uniformly to require the participation of caspases²⁰, the particular caspases necessary vary according to cell-type and the stimulus triggering cell death^{21,22}. Therefore, the ability of each IAP family member to inhibit apoptosis may also vary, depending on the cell and the stimulus involved. XIAP, for instance, may be an effective inhibitor only of apoptotic stimuli that depend on caspase-3 and/or caspase-7. Further experiments will determine in which cell-types and under which apoptosis-stimulating conditions XIAP effectively prevents apoptosis. □

Methods

Expression and purification of proteins. A XIAP-encoding cDNA was obtained by RT-PCR using a first-strand cDNA derived from Jurkat cells as the template and specific primers based upon Genbank accession number U32974 (forward primer, 5'-GGGAATTCATGACTTTTAAACAGTTTGAAGGAT-3'; reverse primer, 5'-CTCTCGAGCATGCCTACTATAGAGTTAGA-3'). The PCR product was digested with *Eco*RI and *Xho*I, followed by ligation into pcDNA3 (Invitrogen) containing an N-terminal Myc tag and into pGEX4T-1 (Pharmacia). Plasmid pGEX4T-1-XIAP was then introduced into an *E. coli* strain BL21(DE3) harbouring the plasmid pT-Trx²³. The GST-XIAP fusion protein was prepared from the soluble fraction upon induction with 0.2 mM IPTG at 30 °C for 3 h, affinity-purified using glutathione-Sepharose, and then dialysed against PBS.

Full-length cDNAs encoding caspases 3, 6 and 7, and a cDNA encoding the catalytic subunits of caspase-8 (Ser 216 → C terminus) were subcloned into

pET vectors (Novagen), expressed in *E. coli* strain BL21(DE3)pLysS as His₆-tagged proteins, and purified as described^{14,16,17}. The preparation of recombinant control proteins, GST-Bcl-2, GST-Bax, GST-CD40 cytosolic domain and His₆-S5a proteasome subunit, has been described^{24–26}.

Enzyme assays. Caspase activity was assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) or *p*-nitroaniline (pNA) from YVAD- or DEVD-containing synthetic peptides using continuous-reading instruments as described¹⁷. Inhibition rates and equilibria were determined by progress curves in which DEVD-AFC hydrolysis was measured using ~0.1 nM caspases and a range of concentrations of rXIAP (0.1–12 μM). *K_i* was calculated without any assumption of the inhibitory mechanism, and therefore without adjustment for the 0.1 mM DEVD-AFC substrate concentration¹⁷. The specific activities of the enzymes (*k_{cat}*/*K_m*) were estimated using Ac-YVAD-AFC for caspase-1 and Ac-DEVD-AFC for all others, and ranged from $1.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for caspase-8 to $1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for caspase-3 (Q. Zhou and G.S.S., manuscript in preparation).

Preparation and induction of the cell-free apoptotic system. Cell extracts were prepared as described⁸, but with several modifications, using 293 embryonic kidney or Jurkat T cells. Cells were washed with ice-cold buffer A (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 0.1 mM PMSF) and suspended in one volume of buffer A. Cells were incubated on ice for 20 min and then disrupted either by passage through a 26-gauge needle 15 times (293 cells) or Dounce-homogenized 15 times in 2 ml buffer using a B-type pestle (Jurkat T-cells). Cell extracts (10–15 mg total protein per ml) were clarified by centrifugation at 16,000g for 30 min and the NaCl concentration increased by 50 mM. For initiating caspase activation, 1 μM horse heart cytochrome *c* (Sigma) and 1 mM dATP were added. Nuclei were prepared from HeLa cells¹⁵ and stained with 0.1 μg μl⁻¹ of acridine orange or ethidium bromide.

Immunoblot analysis. Immunoblotting for caspases was done as described, using 750 mM Tris/12% polyacrylamide gels, after normalizing cell lysates for protein content^{27,28}. Antiserum specific for XIAP was prepared in rabbits using a synthetic peptide NH₂-CDAVSSDRNFPNSTNLPRNPS-amide (amino acids 241–261) conjugated to maleimide-activated keyhole limpet haemocyanin and ovalbumin carrier proteins (Pierce).

Protein-binding assays. GST-XIAP (~3 μg) or GST-CD40 (~6 μg) immobilized on glutathione-Sepharose (5 μl) was added to 50-μl cytosolic extracts (preincubated with or without 1 μM cytochrome *c* and 1 mM dATP for 60 min at 30 °C) or to purified caspases (0.5 μg caspase 3, 6 or 7) in 100 μl caspase assay buffer¹⁶ with 0.1% (w/v) BSA. After incubation at 4 °C for 60 min, beads were removed by centrifugation and washed twice with 100 volumes of 50 mM Tris, pH 7.5, 150 mM KCl, 2 mM DTT, 0.025% Triton-X100 before SDS-PAGE and immunoblot assay.

Transfection of cultured cells. Subconfluent 293T cells were transfected in 6-cm dishes using the calcium phosphate method with 1 μg pcDNA3-human-Bax and either 9 μg control plasmid pcDNA3 or pcDNA3-Myc-XIAP. Transfection efficiency was 80–90% (based on X-gal staining following co-transfection of pCMV-βGal). After culturing for 24 h, both the floating and attached cells were collected and the percentage of dead cells was determined by either trypan-blue or propidium iodide (PI) dye exclusion. Apoptotic cells containing subdiploid DNA were quantified by FACS analysis of PI-stained, ethanol-fixed cells. The remaining cell pellets were lysed in 10 mM HEPES, pH 7.5, 142 mM KCl, 1 mM EGTA, 1 mM DTT, 0.2% NP-40, 0.1 mM PMSF and used for either immunoblot analysis or protease assay.

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***Drosophila* Mad binds to DNA and directly mediates activation of *vestigial* by Decapentaplegic**

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The TGF- β (transforming growth factor- β)-related signalling proteins, including Decapentaplegic (Dpp) in *Drosophila* and bone morphogenic proteins and activin in vertebrates, affect the growth and patterning of a great variety of structures. However, the mechanisms by which these ligands regulate gene expression are not understood. Activation of complexes of type I with type II receptors results in the phosphorylation and nuclear localization of members of the SMAD protein family^{1–9}, which are thought to act as co-activators of transcription, perhaps in conjunction with

sequence-specific cofactors¹⁰. Here we show that the amino-terminal domain of the *Drosophila* Mothers against dpp protein (Mad), a mediator of Dpp signalling^{11–14}, possesses a sequence-specific DNA-binding activity that becomes apparent when carboxy-terminal residues are removed. Mad binds to and is required for the activation of an enhancer within the *vestigial* wing-patterning gene in cells across the entire developing wing blade. Mad also binds to Dpp-response elements in other genes. These results suggest that Dpp signalling regulates gene expression by activating Mad binding to target gene enhancers.

In the *Drosophila* wing imaginal disc, the anteroposterior and dorsoventral compartment boundaries are important signalling sources, and inputs from both axes are required for appendage formation¹⁵. Growth and patterning along the anteroposterior axis depends upon the sequential organizing activities of the Engrailed (En), Hedgehog (Hh) and Decapentaplegic (Dpp) proteins^{16–19}. Dpp acts as a morphogen from its source to organize wing growth and anteroposterior patterning and to activate gene expression over a long range^{19–24}. The *spalt* (*sal*)²⁵ and *optomotor-blind* (*omb*)²⁶ genes are expressed in nested patterns centred on and extending up to 20 cell-diameters away from the stripe of Dpp expression^{22,23} (Fig. 1a). The *vestigial* (*vg*) gene is even more broadly expressed and is required in all cells of the developing wing (Fig. 1a). Activation of *vg* is regulated by signals from both axes through separate *cis*-regulatory elements that control complementary patterns of gene expression²⁴. The 'boundary' enhancer is activated along the dorsoventral boundary through components of the Notch pathway, whereas the 'quadrant' enhancer is activated in the remainder of the developing wing blade (Fig. 1e) by Dpp as well as a signal from the dorsoventral boundary²⁴.

Because Mad is an intracellular signal transducer downstream of Dpp receptors^{1,13,14}, we examined the requirement of Mad activity for *Vg* expression in the wing imaginal disc in mitotic clones with reduced Mad function. Homozygous clones for the strong *Mad*^{1,2} allele in developing wing-blade cells had significantly reduced levels of *Vg* expression and showed a growth disadvantage in comparison with surrounding heterozygous or wild-type cells (twin spot) (Fig. 1b–d, arrows). In contrast, *Mad*^{1,2} clones along the dorsoventral boundary did not show any changes in *Vg* expression levels (Fig. 1b–d, arrowheads). The different effects of the reduction of Mad activity in dorsoventral boundary versus wing-blade clones is explained by the different regulatory elements that control the expression of the *vg* gene in these regions. Mad has no effect on the *Notch*-dependent dorsoventral boundary enhancer, but the quadrant enhancer required Dpp signalling²⁴ and Mad function.

To investigate whether Mad exerts a direct effect on *vg* transcription, we tested whether Mad could bind specifically to the quadrant enhancer. SMAD family members share highly conserved amino- and carboxy-terminal domains, termed MAD homology regions 1 and 2 (MH1 and MH2)², which are separated by a less conserved proline-rich linker region. We found that the Mad MH1 plus linker, expressed as a glutathione S-transferase (GST) fusion protein (designated Mad^N), bound DNA and protected a single interval within the quadrant enhancer in a DNase I footprinting experiment (Fig. 2a); GST and the GST–MAD linker plus MH2 fail to bind DNA (data not shown). The specificity of Mad binding to these protected sequences was demonstrated by a gel mobility-shift assay (Fig. 2b). A 39-base-pair double-stranded oligonucleotide of the *vg* quadrant enhancer containing the Mad-protected region, the Q⁺ probe, was bound readily by Mad^N (Fig. 2b, lane 2). A double-stranded oligonucleotide in which 12 bp of the Mad^N protected region was replaced with two *Bgl*II restriction sites, the Q^m probe, was bound with much lower affinity than the wild-type sequence (Fig. 2b, lane 7). Moreover, the Q^m mutant oligonucleotide did not compete as efficiently as the Q⁺ oligonucleotide with the ³²P-labelled Q⁺ probe (Fig. 2b, compare lanes 3 and 4 with lanes 5 and 6). These data suggest that the binding of Mad^N protein to the quadrant enhancer

is specific. Only a single predominant band shift was observed, regardless of competitor concentration (Fig. 2b, lanes 2–6). These results suggest that Mad^N binds to DNA as a single species.

To test whether the Mad^N binding site is essential for the activation of the quadrant enhancer *in vivo*, we generated transgenic fly lines carrying a *lacZ* reporter gene under the control of the quadrant enhancer in which the 12 bp of the footprinted interval were replaced with one or two 6-bp *Bgl*II restriction sites, the 12-bp substitution corresponding in sequence to the Q^m oligonucleotide that fails to bind Mad^N. Normally, the quadrant enhancer is activated in early third-instar discs as a small patch of expression

near the intersection of the anteroposterior and dorsoventral boundaries and gradually fills in the entire wing pouch by the mid–late third instar²⁴ (Fig. 2c). In contrast, expression of the *lacZ* reporter gene driven by either mutated quadrant enhancer was not detectable in most early to mid third-instar discs. When detected, expression was only observed at a very low level near the antero-posterior boundary (Fig. 2d, e). These data, together with the *in vitro* data from the Mad binding experiments on the *vg* quadrant enhancer, demonstrate that Mad directly mediates the Dpp-dependent transcription of the *vg* gene.

The identification of an essential Mad binding site in the quad-

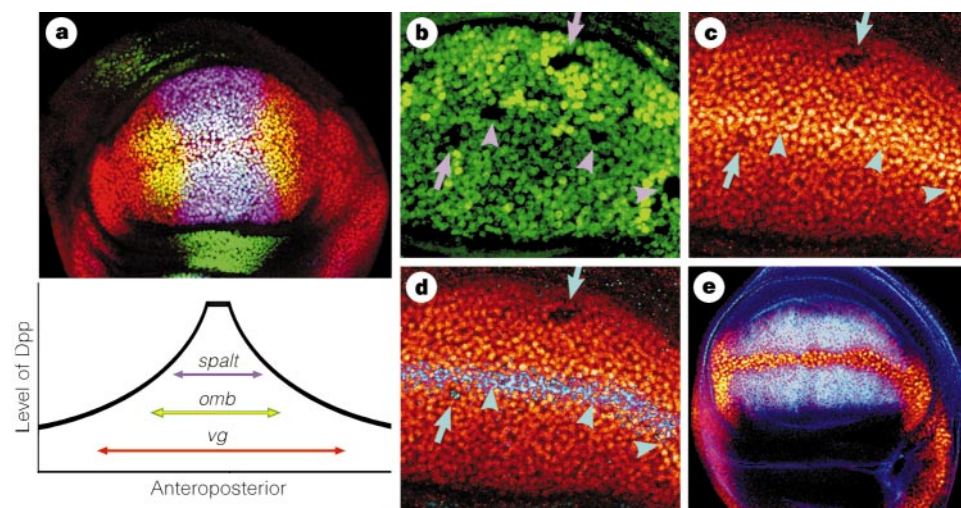


Figure 1 Mad activity is required for Vg expression in the cells of the developing wing blade. Native Vg protein expression is shown in red. **a**, Top, triple-labelled third-instar wing disc with nested domains of Spalt (purple and light blue) and Omb (green), and yellow where coexpressed with Vg expression within the broad Vg (red) domain. Bottom, these wing patterning genes are activated over different distances from the Dpp source. **b**, Mitotic clones homozygous for the hypomorphic *Mad*¹² allele lack expression of the Myc epitope tag (green). **c**, Vg expression in the same disc as **b**. **d**, Wingless (Wg) (blue) expression merged with the Vg expression (red) shown in **b** marks the dorsoventral boundary. The level of Vg expression is reduced only in the *Mad*¹² clone in the future wing blade (arrows), but not in the clones on the dorsoventral boundary (arrowheads). **e**, Vg expression (red) in the future wing-blade cells is regulated by the *vg* quadrant enhancer (β -galactosidase expression driven by the quadrant enhancer is in light blue and purplish pink).

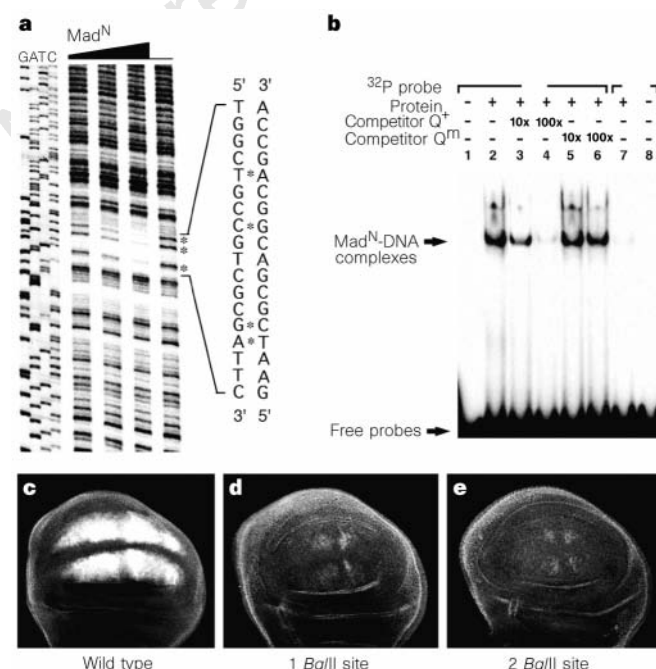


Figure 2 The binding of Mad^N protein to the *vg* quadrant enhancer is sequence specific and essential for activation *in vivo*. **a**, DNase I footprint of the Mad^N protein to the *vg* quadrant enhancer. An autoradiogram of a denatured polyacrylamide gel used to separate the products of DNase I digestion of Mad^N-*vg* quadrant enhancer complexes is shown (right four lanes) with the DNA sequencing products of the *vg* quadrant enhancer (left four lanes, marked with their corresponding ddNTP reaction). The schematic representations of the relative amounts of Mad^N protein used in the reaction (over the right four lanes) shows that Mad^N was increased twofold in the second lane and fourfold in the third lane; the fourth lane is the no-protein control. The DNase I-sensitive bases protected by Mad^N are marked with asterisks. The double-stranded sequence spanning the protected region is shown. **b**, Competition gel mobility-shift assay for the binding specificity of Mad^N to the *vg* quadrant enhancer DNA. The presence or absence of each component is indicated by + or -; 10x and 100x indicate the relative molar excess. Q⁺ is the wild-type double-stranded oligonucleotide containing the Mad^N footprinted interval; Q^m is the oligonucleotide in which the footprinted interval has been replaced with two *Bgl*II sites (see Methods for sequences). The Q⁺ probe was bound well by Mad^N (lane 2) but the Q^m probe was not (lane 7). The unlabelled Q^m oligonucleotide did not compete efficiently for the Q⁺-Mad^N complex formation (lanes 5 and 6), whereas the Q⁺ oligonucleotide did (lanes 3 and 4). **c**, Expression of β -galactosidase driven by the wild-type *vg* quadrant enhancer in a mid–late third-instar wing imaginal disc. **d**, **e**, Expression of β -galactosidase driven by the mutated *vg* quadrant enhancer in which the 12-bp Mad binding site has been replaced with a *Bgl*II site (**d**) and two tandem *Bgl*II sites as in Q^m (**e**). The expression level of β -gal was greatly reduced in these discs compared with the wild-type enhancer (**c**). The imaginal discs in **c–e** carry one copy of the reporter gene.

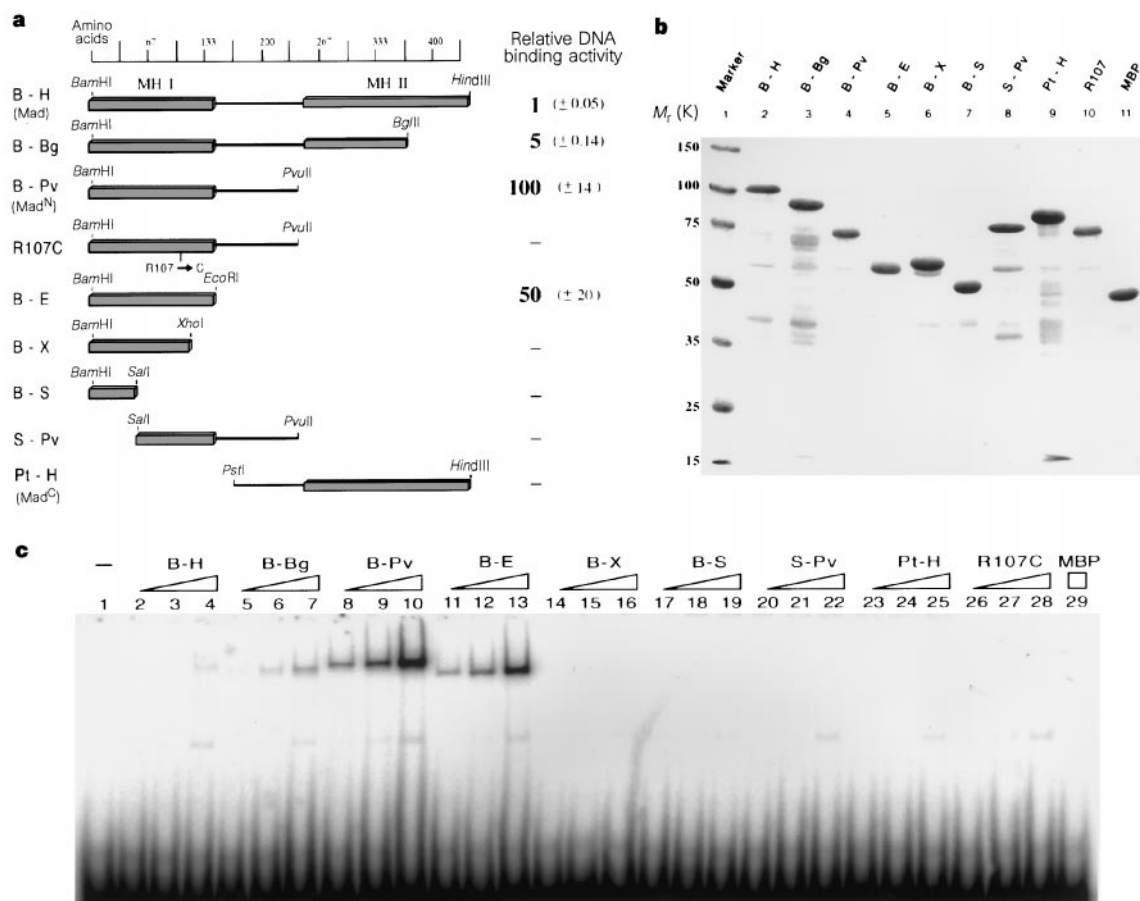


Figure 3 The N-terminal domain of Mad protein (Mad^N) binds to the *vg* quadrant enhancer. **a**, MAD fusions to the C terminus of MBP or GST for use in DNA-binding assays. Conserved MH1 and MH2 regions are shown as boxes, with the intervening linker region indicated by a line. Fusion proteins contain the following MAD amino acids: B-H (Mad), 1–455; B-Bg, 1–372; B-Pv (Mad^N), 1–241; R107C, 1–241; B-E, 1–159; B-X, 1–120; B-S, 1–48; S-Pv, 49–241; Pt-H (MAD^C), 167–455. Amino-acid residue number is indicated at the top. Relative DNA binding activity is shown on the right. Protein concentrations were estimated by display on the SDS

polyacrylamide gel in **b**. Data are from **c** and two repetitions of this experiment. Standard error is indicated within brackets. **b**, Coomassie blue-stained 10% SDS gel showing affinity-purified preparations of MBP fusion proteins used in **c**. **c**, Autoradiographic image of a gel mobility-shift assay testing the ability of MBP-Mad fusions to bind to a ³²P-end-labelled 20-bp *vg* double-stranded oligonucleotide. Lane 1, no protein control; lane 24, unfused MBP; remaining sets of lanes contain progressive 5-fold increases in the proteins labelled as in **a**.

rant enhancer allowed us to localize the Mad DNA-binding domain more precisely. In a series of fusions to the maltose-binding protein (MBP) of *Escherichia coli*, the effects of deletions from the Mad C and N termini (Fig. 3a, b) on DNA-binding activity were tested using an oligonucleotide containing the Mad site of the quadrant enhancer (Fig. 3c). The specific DNA-binding activity of full-length Mad protein (B-H, lanes 2–4) was approximately 100-fold lower than that of C-terminally truncated Mad^N (B-Pv, lanes 8–10); removal of only a portion of MH2 (B-Bg, lanes 5–7) resulted in an intermediate activity. These results show that MH2 effectively inhibits DNA binding and suggests a mechanism that might contribute to inactivation of Mad in the absence of Dpp signalling. Removal of the linker region from the C-terminal end of MH1 (B-E, lanes 11–13) caused a slight reduction in binding activity. Without MH1, MH2 plus linker region (Mad^C) failed to bind to DNA (Pt-H, lanes 23–25). Removal of the C-terminal (B-X, lanes 14–16) or N-terminal (S-PV, lanes 20–22) portion of the MH1 domain abolished DNA-binding activity. Binding was also abolished by a single amino-acid substitution in Mad (Arg107 → Cys) that is the same as that found at position 133 in the Smad2 protein in a colorectal tumour⁴ (R107C, lanes 26–28), demonstrating that this conserved residue is essential for MH1 DNA-binding activity.

We also sought to determine a consensus sequence recognized by

Mad^N. As well as the *vg* quadrant enhancer, several other Dpp-responsive enhancers have been characterized, such as the *labial* endoderm enhancer^{27,28} and the *Ultrabithorax* midgut enhancer, for which the Dpp response element has been localized to the 95-bp DI–DII interval²⁹. Gel shifts with oligonucleotides representing portions of these enhancers (Fig. 4b, lanes 1–18), and the competition of Q⁺ but not Q^m for these oligonucleotides (Fig. 4c, lanes 9–12, 15–18), show that at least one high-affinity Mad^N binding site is found in each, together with multiple low-affinity sites. Alignment of these sequences and comparison of the relative strength of Mad^N binding were used to derive a Mad^N binding-site consensus of GCCGnCGC. The two sites of highest affinity match this consensus perfectly, and three lower-affinity sites contain mismatches at one to three positions (Fig. 4d).

Our results demonstrate that the Mad MH1 region has a sequence-specific DNA-binding domain, and indicate that this DNA-binding activity is important in directing transcriptional activation in response to Dpp signalling. The C-terminal MH2 region, in addition to its previously demonstrated function as a transcriptional activation domain (in the case of Smad1)³, inhibits the DNA-binding activity of MH1 *in vitro*. It is not yet known whether the masking of DNA-binding activity (in addition to cytoplasmic sequestration by receptors⁸) is involved in the regula-

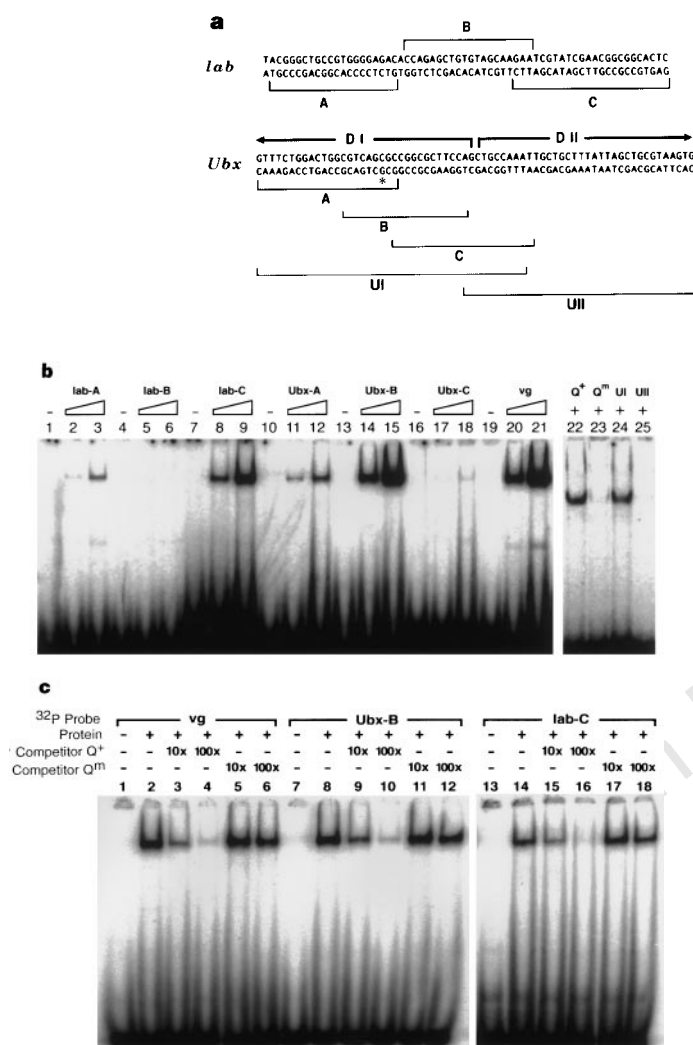


Figure 4 The *lab* endodermal and *Ubx* midgut enhancers contain *Mad^N* binding sites similar to those in the *vg* quadrant enhancer. **a**, DNA sequences of a portion of the *lab* endodermal enhancer and of the D I and D II regions of the *Ubx* midgut enhancer, each of which contains sequences resembling the *Mad* binding site of the quadrant enhancer. The regions corresponding to the probes used for the gel mobility-shift assay in **b** are marked with brackets. The asterisk indicates a base pair that was omitted in the *Ubx-A* probe. **b**, Gel mobility-shift assay identifies a high-affinity *Mad^N* binding site in the *lab* enhancer and one in the *Ubx* D I region. The sequences of the *lab* and *Ubx* probes are shown in **a**. *vg* is a 20-bp probe containing the *Mad^N* binding site; *Q⁺* and *Q^m* are 40-bp probes containing wild-type and mutated versions of the same site. Each set of 30- μ l binding reactions contained (left to right) 0, 100 and 500 ng of fusion protein, as indicated by the minus sign and wedge. **c**, Competition assays for specificity of *Mad^N* binding to *vg*, *Ubx-B* and *lab-C* oligonucleotides. A10- and 100-fold excess of *Q⁺* or *Q^m* oligonucleotide over labelled *vg*, *Ubx-B* or *lab-C* probe was incubated in binding reactions containing 250 ng of *Mad^N*. In each case *Q⁺*, but not *Q^m*, competed efficiently for binding to *Mad^N* showing that each site is bound with a high degree of sequence specificity. **d**, Alignment of sequences that are bound by *Mad^N* in **b**. The GCCGnCGc consensus derived from the aligned sequences matches the two highest affinity sites perfectly, contains one mismatch with a moderate affinity site, and contains 2–3 mismatches with two low-affinity sites.

tion of *Mad* activity by *Dpp* *in vivo*. One further mechanistic issue concerns the establishment of nested expression domains of *Dpp*-regulated genes in the wing. Different thresholds for response to *Dpp* could result from different affinities and/or numbers of *Mad* binding sites in target enhancers. This possibility can be tested once the *Dpp*-responsive *cis*-regulatory element(s) of other wing-patterning genes is isolated and characterized.

Note added in proof: Ersh *et al.* (EMBO J. 16, 2014–2022; 1997) have recently provided evidence that *Dpp* induces visceral mesodermal expression of *Ubx* by direct binding of dCREB B protein to a CRE element. This CRE is adjacent to a palindromic sequence, termed FP5, which footprints with an embryonic nuclear extract. FP5 is coincident with the high-affinity *Mad* binding site we identified in the *Ubx* B oligo. Ersh *et al.* showed that reporter gene response to *Dpp* was dramatically reduced by mutation of the CRE but not by mutation of FP5. Thus *Dpp*-dependent activation of *Ubx* may not require DNA contact by *Mad*, but rather by dCREB B. These contrasting results for *vg* and *Ubx* suggest that the mechanism by which *Mad* mediates transcriptional regulation may vary significantly among the many targets of *Dpp* signalling. □

Methods

Generation of *Mad* clones and immunohistochemistry. Mitotic clones homozygous for the *Mad^{1,2}* allele were generated by heat-shock induction of FLP recombinase in the progeny of *w; Mad^{1,2}, P[ry+, hs-neo, FRT] 40A/CyO* males (stock provided by V. Wiersdorff, M. Mlodzik and S. Cohen) crossed to *w¹¹¹⁸, hsFLP1; P[w+, hs-pM] 36F, P[ry+, hs-neo, FRT] 40A* female flies. Clone

induction and antibody staining of imaginal discs was performed as previously described²⁴.

Production and purification of *Mad* fusion proteins. For fusions containing the MH1 domain (*Mad* and *Mad^N*) a *Bam*HI site was introduced by polymerase chain reaction (PCR) 15 bp upstream of the predicted initiator ATG. Fusion of this *Bam*HI site to a *Bam*HI polylinker site at the C terminus of GST or MBP resulted in insertion of the sequence Gly-Ser-Ser-Asn-Arg between GST (or MBP) and *MAD*. The full-length *Mad* fusions contained *Mad* sequences extending from the *Bam*HI site to a *Hind*III site located within the 3' untranslated region. Truncated fusions were constructed using the restriction sites indicated in Fig. 3a. The R107C point mutation was introduced into the *Mad* coding region by two-primer site-directed mutagenesis, and was confirmed by sequencing. The GST and MBP fusion proteins were purified by affinity chromatography³¹.

Gel mobility shift and DNase I footprinting assays. DNA-binding assays for *Mad* fusion proteins were performed in binding buffer (25 mM Tris, pH 7.5, 80 mM NaCl, 35 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol (v/v), 50 μ g ml⁻¹ poly(dI.dC), 50 μ g ml⁻¹ poly(dA.dT), 150 μ g ml⁻¹ nonspecific competitor DNA) and separated in 5% polyacrylamide gels containing 0.5 $\times$ TBE. The top strands of the double-stranded oligonucleotides used are indicated in Fig. 4 except for the following, in which underlining shows where *Q⁺* and *Q^m* differ in sequence: *Q⁺*, TTTGTGCTTGCTGCCGTCGCGATTTCG ACAACTTTGG; *Q^m*, TTTGTGCTTGAGATCTAGATCTATTGACAACITTT GG; *Vg*, CTTGGCTGCCGTCGCGATTTC. Oligonucleotide probes corresponding to regions of the *vg* quadrant, *Ubx* midgut, and *lab* endodermal enhancer sequences were labelled with [γ -³²P]ATP (6,000 Ci mmol⁻¹, NEN) and T4 polynucleotide kinase. The probe DNAs for DNase I footprinting were

synthesized by PCR. DNase I footprinting reactions were processed as described²⁴.

Reporter gene construction. Substitution of a single *Bgl*II site for the quadrant enhancer Mad binding-site sequence GCTGCCGTCGCG was done by assembling two fragments generated by PCR with *Pfu* polymerase. Expansion of the single *Bgl*II site to two tandem sites matching the Q^m sequence was done by insertion of an oligonucleotide containing *Bbs*I internal sites that allowed for subsequent cleavage and ligation to generate the tandem *Bgl*II sites. Both sequences were confirmed by sequencing and ligated into the *Kpn*I/*Not*I sites of the Hsp *lacZ*-CaSpeR *Drosophila* transformation vector for the generation of transgenic lines²⁴.

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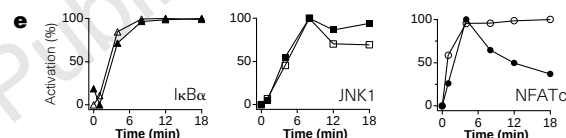
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Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration

Ricardo E. Dolmetsch, Richard S. Lewis, Christopher C. Goodnow & James I. Healy

Nature **386**, 855–858 (1997)

The graphs in Fig. 2e were accidentally swapped around during the production process. The correct versions are shown here. □



Large-scale tectonic deformation inferred from small earthquakes

Falk Amelung & Geoffrey King

Nature **386**, 702–705 (1997)

Owing to an error in the production process, a length scale was omitted from Fig. 2b and the shading in Fig. 2d did not show up. The corrected figure is shown here. □

